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CONTENTS OF VOL. 79.

No. 1. *July* 28, 1933.

PAGE

The effect of gonadectomy on the adrenal, thyroid, and pituitary glands. By DOROTHY H. ANDERSEN and HELEN S. KENNEDY 1

Diffusion of inorganic phosphate into and out of the skeletal muscles and bones of the frog. By M. GRACE EGGLETON . . 31

Carotid sinus reflexes. Influence of central blood-pressure and blood supply on respiratory and vaso-motor centres. By J. J. BOUCKAERT and C. HEYMANS 49

The action of veratrine, curare and strychnine on the response of medullated nerve. By HANS FROMHERZ 67

The effect of quaternary ammonium salts upon nerve. (Preliminary communication.) By S. L. COWAN and H. R. ING . . 75

The reactivity and activity of the human uterus at various stages of pregnancy and at parturition. By J. M. ROBSON . . 83

Choline and liver fat in diabetic dogs. By C. H. BEST, G. C. FERGUSON and J. M. HERSHEY 94

Reversible inexcitability of tactile endings in skin injury. By T. P. FENG 103

The influence of temperature on the rate of excretion of phenol-sulphonephthalein in the frog. By E. M. SCARBOROUGH. . . 109

The histological changes in the bone responsible for the action of parathyroid hormone on the calcium metabolism of the rat. By L. I. PUGSLEY and HANS SELYE 113

Capacity of the uterus of the rabbit to respond to prolonged luteal activity. By M. K. MCPHAIL 118

No. 2. *September* 4, 1933.

The physiological significance of Weber's law and colour contrast in vision. By J. S. HALDANE 121

The effect of œstrin on the reactivity and spontaneous activity of the rabbit's uterus. By J. M. ROBSON 139

A simple photoelectric colorimeter. By G. A. MILLIKAN . . 152

PAGE

The kinetics of blood pigments: hæmocyanin and hæmoglobin. By G. A. Millikan 158

Ovulation after blood dilution and cross-circulation. By M. K. McPhail, A. S. Parkes and W. E. White 180

A note on the retinal action potential of the human eye. By Sybil Cooper, R. S. Creed and Ragnar Granit 185

Albuminuria in the normal male rat. By Muriel E. Bell . 191

The diffusion of lactate into and from muscle. By S. C. Devadatta 194

An isolated mammalian heart preparation capable of performing work for prolonged periods. By I. de Burgh Daly and W. V. Thorpe 199

The influence of the parathyroid on the metabolism of creatine and phosphoric acid. By C. G. Imrie and Constance N. Jenkinson 218

The excretion of prolan after intravenous injection into the rabbit. By A. S. Parkes and W. E. White 226

The extra-uterine survival of spermatozoa. By W. E. White . 230

Histamine in canine gastric tissues. By Gertrude Gavin, E. W. McHenry and M. J. Wilson 234

No. 3. *October* 6, 1933.

The effect of atropine on adrenaline hyperglycæmia in rabbits decerebrated anterior to the pons. By M. G. Forster (assisted by A. K. Chalmers) 239

The alleged occurrence of acetylcholine in ox blood. By Harold Ward Dudley 249

Choline esters in tissue extracts. By H. C. Chang and J. H. Gaddum 255

Insulin and adrenaline. By M. W. Goldblatt 286

The normal behaviour of the isolated uterus of the guinea-pig, and its reactions to œstrin and oxytocin. By W. H. Newton . 301

The effect of the cardiac contraction upon the coronary flow. By G. V. Anrep and E. v. Saalfeld 317

Afferent impulses in the vagus and their effect on respiration. By E. D. Adrian 332

No. 4. *October* 25, 1933.

PAGE

The renal elimination of injected urea and creatinine. By W. W. Kay and H. L. Sheehan 359

The control of the insulin output of the pancreas. By Takao Kosaka 416

The outline method for investigating gastric motility. By B. A. McSwiney and W. R. Spurrell 423

Influence of osmotic pressure upon the emptying time of the stomach. By B. A. McSwiney and W. R. Spurrell 437

Blood lactic acid in man during rest. By Leslie Colin Cook and Richard Henry Hurst 443

Adrenaline in the suprarenal medulla. By Heinz Schild . . 455

The influence of certain factors upon the volume of the intra-thoracic venæ cavæ. By K. J. Franklin 470

Effect of breathing on the intra-abdominal pressure. By W. H. Wilson 481

The electrical resistance of stimulated muscle. By W. Hartree 487

A revised analysis of the initial heat production of muscle. By W. Hartree 492

The effect of ions on the cutaneous sensory endings of the frog. By M. Talaat 500

The respiratory quotient of the eviscerate cat. By James McInnes Peterson 508

LIST OF AUTHORS.

PAGE

ADRIAN, E. D. Afferent impulses and respiration 332

ANDERSEN, D. H. and KENNEDY, H. S. Effect of gonadectomy on glands 1

ANREP, G. V. and SAALFELD, E. v. Cardiac contraction and coronary flow 317

BELL, M. E. Albuminuria in normal male rats 191

BEST, C. H., FERGUSON, G. C. and HERSHEY, J. M. Choline and liver fat in diabetic dogs 94

BOUCKAERT, J. J. and HEYMANS, C. Carotid sinus reflexes . . 49

CHANG, H. C. and GADDUM, J. H. Choline esters in tissue extracts 255

COOK, L. C. and HURST, H. H. Blood lactic acid in man during rest 443

COOPER, S., CREED, R. S. and GRANIT, R. Action potential of human retina 185

COWAN, S. L. and ING, H. R. Quaternary ammonium salts on nerve 75

CREED, R. S., GRANIT, R. and COOPER, S. Action potential of human retina 185

DALY, I. DE B. and THORPE, W. V. Isolated mammalian heart preparation 199

DEVADATTA, S. C. Diffusion of lactate into and from muscle . . 194

DUDLEY, H. W. Alleged acetylcholine in ox blood 249

EGGLETON, M. G. Phosphate diffusion in frog muscles . . . 31

FENG, T. P. Tactile endings and skin injury 103

FERGUSON, G. C., HERSHEY, J. M. and BEST, C. H. Choline and liver fat in diabetic dogs 94

FORSTER, M. G. Adrenaline hyperglycæmia and atropine . . 239

FRANKLIN, K. J. Volume of intrathoracic venæ cavæ 470

FROMHERZ, H. Veratrine, etc. on nerve 67

GADDUM, J. H. and CHANG, H. C. Choline esters in tissue extracts 255

GAVIN, G., MCHENRY, E. W. and WILSON, M. J. Histamine in canine gastric tissues 234

GOLDBLATT, M. W. Insulin and adrenaline 286

GRANIT, R., COOPER, S. and CREED, R. S. Action potential of human retina 185

HALDANE, J. S. Weber's law and colour contrast 121

HARTREE, W. Electrical resistance of stimulated muscle . . 487

PAGE

HARTREE, W. Initial heat production of muscle 492
HERSHEY, J. M., BEST, C. H. and FERGUSON, G. C. Choline and liver fat in diabetic dogs 94
HEYMANS, C. and BOUCKAERT, J. J. Carotid sinus reflexes . . 49
HURST, H. H. and COOK, L. C. Blood lactic acid in man during rest 443
IMRIE, C. G. and JENKINSON, C. N. Creatine phosphate and parathyroid 218
ING, H. R. and COWAN, S. L. Quaternary ammonium salts on nerve 75
JENKINSON, C. N. and IMRIE, C. G. Creatine phosphate and parathyroid 218
KAY, W. W. and SHEEHAN, H. L. Urea and creatinine elimination 359
KENNEDY, H. S. and ANDERSEN, D. H. Effect of gonadectomy on glands 1
KOSAKA, T. Insulin regulation 416
MCHENRY, E. W., WILSON, M. J. and GAVIN, G. Histamine in canine gastric tissues 234
MCPHAIL, M. K. Prolongation of pseudopregnancy . . . 118
MCPHAIL, M. K., PARKES, A. S. and WHITE, W. E. Ovulation and cross-circulation 180
MCSWINEY, B. A. and SPURRELL, W. R. Gastric motility . . 423
MCSWINEY, B. A. and SPURRELL, W. R. Osmotic pressure and gastric motility 437
MILLIKAN, G. A. A simple photoelectric colorimeter . . . 152
MILLIKAN, G. A. The kinetics of blood pigments 158
NEWTON, W. H. Uterine muscle 301
PARKES, A. S. and WHITE, W. E. Excretion of prolan . . . 226
PARKES, A. S., WHITE, W. E. and MCPHAIL, M. K. Ovulation and cross-circulation 180
PETERSON, J. MCI. Respiratory quotient of eviscerate cat . . 508
PUGSLEY, L. I. and SELYE, H. Bone changes with parathyroid hormone 113
ROBSON, J. M. Reactivity of human uterine muscle . . . 83
ROBSON, J. M. Œstrin and uterine muscle 139
SAALFELD, E. v. and ANREP, G. V. Cardiac contraction and coronary flow 317
SCARBOROUGH, E. M. The influence of temperature on secretion . 109
SCHILD, H. Adrenaline in the suprarenal medulla 455
SELYE, H. and PUGSLEY, L. I. Bone changes with parathyroid hormone 113
SHEEHAN, H. L. and KAY, W. W. Urea and creatinine elimination 359
SPURRELL, W. R. and MCSWINEY, B. A. Gastric motility . . 423

PAGE

SPURRELL, W. R. and MCSWINEY, B. A. Osmotic pressure and gastric motility 437
TALAAT, M. Effect of ions on tactile endings 500
THORPE, W. V. and DALY, I. DE B. Isolated mammalian heart preparation 199
WHITE, W. E., MCPHAIL, M. K. and PARKES, A. S. Ovulation and cross-circulation 180
WHITE, W. E. and PARKES, A. S. Excretion of prolan . . . 226
WHITE, W. E. Spermatozoon survival 230
WILSON, M. J., GAVIN, G. and MCHENRY, E. W. Histamine in canine gastric tissues 234
WILSON, W. H. Intra-abdominal pressure 481

PROCEEDINGS OF THE PHYSIOLOGICAL SOCIETY.

July 1, 1933.

PAGE

Dikshit, B. B. The presence in the brain of a substance resembling acetyl choline . . . . . 1 *P*
Cruickshank, E. W. H. On the significance of the action of insulin on the R.Q. of the diabetic heart . . . . . 2 *P*
Brunton, C. E. The *p*H of the arterial blood during gastric secretion . 4 *P*
Drury, A. N. and Wedd, A. M. The influence of nucleic acid derivatives upon the coronary blood flow in the dog . . . . . 5 *P*
Sinclair, H. M. The effect of vitamin B_1 upon the respiratory quotient of brain . . . . . 6 *P*

LIST OF AUTHORS.

BRUNTON, C. E. *p*H of blood during gastric secretion . . . . 4 *P*
CRUICKSHANK, E. W. H. Insulin on diabetic heart . . . . 2 *P*
DIKSHIT, B. B. Substance like acetyl choline in brain . . . . 1 *P*
DRURY, A. N. and WEDD, A. M. Nucleic acid derivatives on coronary flow . . . . . 5 *P*
SINCLAIR, H. M. Vitamin B_1 on the R.Q. of brain . . . . . 6 *P*
WEDD, A. M. and DRURY, A. N. Nucleic acid derivatives on coronary flow . . . . . 5 *P*

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THE EFFECT OF GONADECTOMY ON THE ADRENAL, THYROID, AND PITUITARY GLANDS.

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Introduction.

In view of the recent increase in our knowledge of the intimate relation between the endocrine glands and reproduction and the contradictory reports in the literature as to changes produced in the endocrine glands by removal of the reproductive organs, it has seemed to us advisable to sift the literature critically and also to obtain new experimental data. The necessity for a uniform diet and for the elimination of infection has not been fully appreciated in most of the earlier work, and the recent application of the statistical method to biological data has brought greater certainty as to the significance of variations from the normal under different experimental conditions. Our observations on the changes in the pituitary and adrenal glands associated with œstrus have provided us with more satisfactory information concerning normal adult female rats than has previously been available.

I. The Adrenals.

(1) *Review of literature.*

The literature on the effect of gonadectomy upon the adrenal gland is confusing, partly because of the variety of results and partly because of the inadequate data in most of the reports. Very few of the workers escaped one or another of the errors inherent in work with the adrenal gland, especially those resulting from disregard of the following points: (1) the wide normal variability in the weight of the adrenal gland necessitating the use of many animals; (2) the changes which accompany various phases of reproduction, as puberty, œstrus, and pregnancy; (3) the age and body weight of the animals used; (4) the presence of accidental infections; (5) the effect of diet; (6) the interval between operation and the sacrifice of the animal.

If the various conditions of the experiments be disregarded for the moment, the conclusions of the authors as to the effect of gonadectomy on the adrenals may be summarized as follows:

(1) Hypertrophy was found: in male rabbits by Schenk [1910], Kolde [1913], Zchwetadse [1927], Leinati [1929], and Livingston [1916]; in female rabbits by Schenk, Kolde, and Leinati; in male guinea-pigs by Leinati and Rigano-Irrera [1929]; in female guinea-pigs by Raineri [1908]; in male rats by Hatai [1915]; in male mice by Altenburger [1924], Masui and Tamura [1926], and Deanesly [1928]; in dogs by Feodosyeff [1905]; and in frogs by Ciaccio [1903].

(2) Transitory hypertrophy was found: in male and female rabbits by Soli [1909]; in male guinea-pigs by Marrassini [1906]; and in guinea-pigs by Soli.

(3) No change was found: in male rabbits by Marrassini and Luciani [1911]; in female rabbits by Marrassini and Luciani, Dick and Curtis [1912], or by Livingston [1916]; in male and female guinea-pigs by Marrassini and Luciani; in immature female mice by Deanesly [1928]; in mature female mice by Howard-Miller [1927]; in male and female dogs by Pianese [1929]; and in male sheep, oxen and capons by Marrassini and Luciani [1911].

(4) Slight atrophy was reported in female rats by Hatai [1915]; in female mice by Masui and Tamura; in male rats by Minowada [1928]; in adult female mice by Deanesly [1928]; and in capons by Soli [1909].

A critical survey of the work on each species is presented.

(1) *Rabbit.* This is the species most often studied. The earliest report on this animal which we have found is that of Soli in 1909. In the course of his work on the effect of castration on the thymus, he castrated seven male and spayed three female rabbits with ten controls paired by age and presumably by sex, and killed 1–6 months after operation. He gave no weights, but stated that the adrenal volume was increased in those killed 1 month after operation and decreased slightly in those killed after longer intervals.

Schenk in 1910 spayed eleven female rabbits, with four controls: four were spayed at the ages of 3–7 weeks with one control, and killed after 109–128 days; three were spayed at the age of 6 months with two controls and killed after 60 days; and four at the age of 9 months or over, with one control, and killed after an interval of 31–114 days. One adult male without a control was also castrated. Schenk gave no body or adrenal weights, but made a histological study of the adrenals and

reported uniform results, with hypertrophy of the cortex, enlargement of the cells of the zona fasciculata, and a slight decrease in the size of the medulla. The inadequacy of the controls and the lack of standardization cast doubt on the conclusions. Marrassini and Luciani [1911] castrated seven male and spayed six female rabbits of various ages with an equal number of controls paired by sex and weight. The animals were killed after intervals of 15–90 days, and no constant change in the adrenals was observed, although the variations were great. Dick and Curtis [1912] spayed nine adult female rabbits with six controls, and killed them 30–100 days after operation. They recorded the weights of the adrenals and of the eviscerated animals, both of which varied greatly, but found the same mean relative adrenal weight in each series, namely 0·32 mg. per g. of body weight. They made histological studies of the adrenals and found no difference between those of the two series. Kolde [1913] spayed eight females and castrated two males, at the ages of 3–6 months and killed them after 3–6 months. As controls he used five normal males, eleven non-pregnant females and five pregnant females of various ages from 4 months to several years. He gave no weights, but stated that sections of the adrenals of the castrated rabbits appeared larger than those of the normal ones.

Livingston [1916] studied twenty-one male rabbits castrated at a body weight of 430–220 g. with sixteen controls, and nine female rabbits spayed at a weight of 730–2125 g. with eleven controls. He killed these animals after intervals of 99–130 days, when the average body weight was approximately 2 kg. He found a slight but irregular increase in weight in the adrenals in the castrated males, but no change in the spayed females as compared with controls of the same sex.

Zchwetadse [1927] castrated five male rabbits and removed one testis from each of two more. He had one control. The ages were not reported, but the body weights at operation varied from 845 to 3825 g. The animals were killed at intervals of 1½–4 months. The adrenals were not weighed but were sectioned, and changes in both medulla and cortex were found in all cases. The cortical changes varied with the interval after operation, with a temporary increase and later a decrease in the size of the cells of the fasciculata. Leinati [1929] used six castrated rabbits with three controls and one spayed female with one control. Three of the males and two controls were 3-months-old litter mates, killed at intervals of 10, 20, and 30 days respectively. He reported cortical hypertrophy, but gave no body weights and gave adrenal weights in round numbers, so that their accuracy is open to question. The figures indicate some

hypertrophy. The two females were of the same litter, and after 40 days the spayed one had larger adrenals than the control. The remaining three castrated and one control male rabbits were adults, the interval was 5–6 months, and examination of the figures does not reveal the hypertrophy which he reported, if the body weight be considered. He also stated that accessory adrenals were hypertrophied and therefore easier to find in the castrated animals.

Since no one of these workers has an adequate series at constant conditions of age and post-operative interval, and the only series in which the animals and adrenals were weighed (namely that of Marrassini and Luciani [1911]) showed such extreme variations in the weights of the adrenals, we may conclude with these workers that no constant change has been demonstrated in the adrenal glands of the rabbit following gonadectomy.

(2) *Guinea-pig.* The earliest experiments of the kind on the guinea-pig which have been found are those of Marrassini in 1906. He castrated twenty males with fourteen male controls, the body weights at death varying from 320 to 650 g. The weights are approximately parallel in the two series. He killed the operated animals at various intervals, four after 4 days, five after 8 days, three after 12 days, five after 15 days, and three after 24 days, weighed both the adrenals and the guinea-pigs and calculated the weight of the adrenal in percentage of that of the body. There was a marked increase in the relative weight for the first 16 days, with the highest weight at 8 days, namely 0·064–0·078 p.c., followed by a consistent decrease to 0·0487–0·052 p.c. in the 24-day group. This is still higher than the controls which vary from 0·033 to 0·045 p.c. Histologically this hypertrophy was found due to an increase in the vacuoles and size of cells in the fasciculata. This is the longest as well as the most carefully controlled series reported on the guinea-pig. In 1911 Marrassini and Luciani noted the adrenal weights in the course of a series of experiments on the effect of castration on the hypophysis. In six castrated male and eight spayed female guinea-pigs with an equal number of controls paired by age and sex and killed at intervals of from 3 to 90 days, the adrenal weights, the ages of the animals and the post-operative intervals varied so greatly that no conclusions can be drawn. The authors include the figures in their tables without discussing them.

Raineri in 1908 spayed a series of female guinea-pigs and killed them after intervals of 4 days to 3 months. He also hysterectomized other animals and performed the double operation on some. No mention of age, weight, or numbers of animals was made. He found a progressive hyper-

trophy with a maximum value of 4x normal after 3 months. Without records of controls this figure appears unreliable, especially in view of Marrassini's more careful work. Soli in 1909 examined four castrated and four control guinea-pigs and reported the same findings as in his experiments with rabbits. The next experiments on guinea-pigs appear to be those of Rigano-Irrera in 1929, who castrated twelve and vasectomized twelve animals with twelve controls, all adults, and killed them at intervals of 48 hours to 350 days. The figures which he gives indicate an increase in adrenal weight during the first month, which is about equal in the castrated and vasectomized animals, and which later drops, but not to the level of the controls, and remains higher in the vasectomized than in the castrated animals. This is also true in the group killed after 200–350 days. No note is made as to the age or weight of the animals at operation nor as to the age of the controls. Leinati [1929] castrated five young guinea-pigs with three controls, with an average weight for all eight of 350 g. He killed them at intervals of 10–80 days and found cortical hypertrophy in all of them, due to hypertrophy of the zona fasciculata. The body weights varied greatly, but calculation of the relative weights from his figures shows what appears to be a definite hypertrophy, which, however, was more marked in those of his animals which were smaller and probably younger. Parhon, Marza and Kahane in 1926 reported the water content of the adrenals in ten castrated male guinea-pigs and their controls which were as near the same age as possible. The animals were sacrificed 1–10 months after castration. In seven pairs the water content was greater in the castrated animals, while in three the reverse was true. The authors concluded that there was a temporary decrease followed by an increase in the water content of the adrenals in the castrated animals. In a survey of the experiments on guinea-pigs one is impressed with the greater care in standardization found in the work of Marrassini, and with his more detailed report of the experiments. His animals are also the most numerous and are in groups of three to five under a given set of conditions, with careful notes as to organ weights. In the work of the other investigators the age of each animal at operation and the interval after operation varies so widely and the normal variations in the adrenal weight are so completely disregarded as largely to invalidate the results. The additional fact that Marrassini's conclusions agree with those of the present authors tempts them to believe him.

(3) *Rat.* Hatai in 1915 reported a series of experiments in rats, as follows: (1) twenty-six males were castrated at 16–22 days and killed after 100–173 days with twenty-seven controls; (2) five males were cas-

trated at 79–93 days and killed after 90–123 days with six controls; (3) eight males were castrated at 208–232 days and killed after 44–60 days with seven controls; (4) twenty-eight females were spayed at 19–30 days and killed after 101–190 days with thirty controls; (5) eight females were spayed at 97–119 days and killed after 98–100 days with nine controls; (6) seven females were spayed at 172–195 days and killed after 27–60 days with nine controls. Instead of comparing the adrenal weights directly, he compared them with the values given for rats of the same body length according to the Wistar Institute tables. He calculated the percentage difference of the adrenal weight of each rat from the adrenal weight given for rats of its body length in these tables. He then obtained the mean of these figures for the percentage difference of all the control rats and the corresponding mean for the castrated rats, and added these two figures to obtain the percentage difference between mean and control rats. This method ignores the possibility of variations in adrenal weight from causes other than those of sex, body length, and castration. No explanation is offered for the fact that the adrenal weights of the control rats differed as much from those given in the standard tables as did the adrenal weights of the operated animals. This difference may well be due to variations in diet, environment, season or degree of infection in his control rats as compared with the rats used for preparation of the tables. Direct calculations from his figures show a much smaller difference between the castrated and control animals in five of the six experiments, than does his method of calculation. The figures are given for comparison:

Series	Mean adrenal weight in g.		p.c. difference by direct comparison	p.c. change (Hatai)
	Castrated	Control		
1	0·0371	0·0344	+ 7·8	+17·6
2	0·0417	0·0421	+ 1·0	+ 6·8
3	0·0362	0·0346	+ 4·6	+ 8·5
4	0·0436	0·0497	−12·3	−18·5
5	0·0401	0·0521	−23·0	−25·0
6	0·0362	0·0391	− 7·2	− 5·3

The difference between the two methods of calculation is significant only in the male series. They are partly based on the fact that his control animals grew to a lesser length than the castrated ones, which is not the universal experience. The old adult controls of Series 6 have much smaller adrenals than the young adult controls of Series 5. This can be explained by our observations that old female rats often have irregular cycles, which we have found to be associated with low adrenal weights [Andersen and Kennedy, 1932]. The different results obtained in Series 5 and 6 can be interpreted as due to age changes in the controls of Series 6 rather

than to age changes in response to gonadectomy. Hatai concluded that the adrenals showed opposite reactions in the two sexes as the result of gonadectomy, with an increase in castrated males and a decrease in spayed females. He also removed one gonad from a series of males and females and found no changes in the adrenals. After ligation of the spermatic cords he found no adrenal changes unless the testes had undergone advanced atrophy, in which case the changes were identical with those found in the castrated animals.

Minowada [1928] observed a slight decrease in weight in the adrenals of male rats after castration, but found no histological changes.

(4) *Mouse.* The mouse has been the object of several studies. Altenburger [1924] found hypertrophy in the adrenals of four male mice castrated at 3 weeks and killed at about 4 months of age, as compared with two controls. Masui and Tamura [1926] castrated thirty mice and spayed thirty more at the age of about 20 days, and had an equal number of controls of the appropriate sexes. They killed them at the age of 90 days and sectioned the adrenals. They did not say that the sections were serial, but measured the square area of a section, presumably through the centre of the gland, with the width of the medulla, reticularis, and the remaining cortical zones. In the male castrates they described a decrease in the width of the fasciculata and glomerulosa, taken together, with an increase in the reticularis. In the females there was a slight decrease in size without structural changes. Howard-Miller [1927] reported no change in the *x* zone after ovariectomy in twelve mice in comparing one adrenal removed at the time of spaying with the other removed 13–33 days later. Deanesly [1928] made a more careful study in a shorter series of fourteen castrated males with fourteen adult male controls and ten spayed females with fourteen adult female controls. The males were castrated at varying ages from 3 to 12 weeks and killed after 2–10 weeks. She found an increase in the adrenals up to 40 p.c. if the operation was performed before puberty, with the appearance of an *x* zone similar to that in the female; this zone is usually atrophic in the adult male. Five of the females were spayed before maturity and examined after 2 and 12 weeks. The adrenals resembled those of the normal adult female. In the five mice spayed after maturity the *x* zone had become partially degenerated at 16 weeks and completely so at 30 weeks after operation. These observations are based on serial sections, not on weights. These various authors are in essential accord as to the effect of gonadectomy on the adrenals in the mouse, namely a hypertrophy in the male, due chiefly to the increase in the *x* zone, more marked in operations on the immature animals, which

may be interpreted as continuation of the immature structure. There is little, if any, change in the female, in which the *x* zone is found in normal immature and mature animals. The presence of the *x* zone in this species makes comparison with other species difficult.

(5) *Dog.* Feodosyeff [1905] reported the results of spaying in female dogs. The age, weight, number of animals, and existence of controls was not mentioned. He killed them at varying intervals up to 9 months and 3 weeks. He gave a minute description of the histological appearance of the adrenals, which consisted of marked hypertrophy of the zona glomerulosa and zona fasciculata with mitoses and increase in the size of the cells. Pianese [1929] examined three castrated male and three spayed female dogs with an equal number of controls of the appropriate sexes. He operated on them at the age of 3 years and a weight of 8–9 kg. and killed them after 1 month. He found no changes in the adrenals. No experiments on cats have been found.

(6) *Other species.* Marrassini and Luciani [1911] recorded the adrenal and body weights on five spayed sheep with four controls, but the age at operation was not given and the age at death was 5–8 years in the controls and 1 year in the castrated animals, so that no conclusions can be drawn. Of nineteen castrated male cattle, eleven were of an age comparable to that of the five normal animals, namely in the range of 3–9 years, and no clear-cut differences in the adrenal weights were observed.

In addition to mammals, experiments have been reported in frogs and chicks. Ciaccio reported hypertrophy of the adrenal to double the normal size in frogs killed 15 days after gonadectomy, the increase being entirely cortical. Soli, in fifteen capons, killed 2–6 months after castration, with fifteen control cocks of the same age, found slight decrease in the volume of the adrenals. Marrassini and Luciani recorded the adrenal weights in nineteen capons and six cocks of widely varying ages and post-operative intervals and no constant change is observed in this series.

(2) *Technique.*

Preliminary experiments were carried out several years ago on young rats which were spayed or castrated between the ages of 50 and 85 days and killed after intervals varying from 1 to 3 months (Table I, Exp. I; Table II, Exp. IX). In these animals the adrenals of the spayed females were markedly atrophied and those of the castrated males were somewhat hypertrophied. Since the change in the latter was quite variable, the experiment was repeated with greater care on a larger series of male animals (Table I, Exp. II). These rats were castrated at the age of 100 days and

TABLE I. The weight of the pituitary, thyroid and adrenals of the rat after castration.

Exp. No.	Castration at age days	Killed after days	Castration, vasectomy or control	No. of rats	Body weight in g.	Weight in mg.			mg./kg. body weight		
						Pituitary	Thyroid	Adrenal	Pituitary	Thyroid	Adrenal
I	53–82	27–80	Castr.	8	222±13	—	19 ±1	32 ±4	—	87·4± 7·3	145·2±12·7
			C.	7	235±12	—	21 ±2	27 ±2	—	91·3± 9·7	116·7± 8·6
II	100	50	Castr.	15	287±23	14·0±1·1	20·9±2·6	35·1±3·3	49·3±5·2	72·8± 6·5	123·7±13·5
			C.	13	297±29	10·6±0·9	25·9±3·2	32·0±3·6	35·9±3·0	88·4±10·7	108·1± 7·7
III	21	7	Castr.	6	63± 2	3·8±0·2	7·9±0·4	16·5±1·0	60·3±2·9	126·1± 7·0	263·1± 9·5
			C.	6	64± 3	3·4±0·3	8·4±0·8	16·8±1·2	52·7±2·3	128·2±10·8	269·0±18·7
IV	21	21	Castr.	4	101± 7	6·1±0·2	11·6±1·7	23·9±2·2	60·4±3·8	113·7±13·0	236·0± 7·3
		21	Vas.	4	102±10	4·4±0·3	11·3±0·6	20·7±2·4	46·5±2·4	112·3± 9·2	203·7±12·4
			C.	4	96± 6	3·9±0·2	11·2±0·8	21·7±1·5	41·3±4·7	116·9± 8·6	225·2±10·1
V	21	64	Castr.	6	214±12	11·4±0·5	20·0±1·3	38·0±4·7	53·4±2·2	93·6± 5·4	176·7±16·0
		64	Vas.	6	232±16	8·6±0·8	26·6±2·5	31·9±3·1	37·2±3·3	114·3± 6·0	137·4± 8·5
			C.	6	227±15	8·5±0·8	24·9±2·6	32·0±4·9	37·7±2·2	111·0±12·7	139·9±15·0
VI	80	7	Castr.	5	215± 7	8·0±0·6	24·5±2·0	31·9±3·4	37·3±2·1	114·1± 8·8	148·7±16·2
		7	Vas.	4	244±12	8·6±0·4	24·9±2·5	31·5±3·2	35·2±0·1	102·9±13·1	129·7±14·5
			C.	4	240±19	8·4±0·4	26·2±3·6	30·9±3·9	35·1±1·1	108·1± 7·1	128·2± 8·0
VII	80	21	Castr.	4	238±14	10·0±0·3	23·4±1·5	29·3±0·4	42·4±1·7	98·8± 6·8	124·1± 6·9
		21	Vas.	4	250± 8	8·7±0·5	34·0±4·9	33·8±1·2	34·6±1·6	134·9±17·5	135·6± 8·2
			C.	4	246± 6	8·3±0·3	32·0±5·4	35·5±1·8	33·6±1·0	129·9±21·9	144·3± 6·8
VIII	80	56	Castr.	4	238±15	12·4±1·6	20·0±1·9	29·5±2·6	53·6±8·3	84·7±10·0	123·9± 6·4
		56	Vas.	4	252±12	9·5±0·6	24·5±1·3	34·1±2·7	37·5±0·7	97·4± 4·6	135·2± 6·4
			C.	4	252± 7	8·6±0·7	25·9±5·7	30·8±1·2	34·1±2·3	102·4±22·7	122·0± 4·9

TABLE II. The weight of the pituitary, thyroid and adrenals of the rat after spaying.

Exp. No.		Spaying at age days	Killed after days	Operation or control	No. of rats	Body weight in g.	Weight in mg.			mg./kg. body weight		
							Pituitary	Thyroid	Adrenal	Pituitary	Thyroid	Adrenal
IX		53–73	30–60	Op.	12	203 ± 9	—	15 ±2	33 ±4	—	73·4 ± 8·3	162·8 ± 18·9
				C.	11	179 ± 13	—	20 ±3	40 ±6	—	110·2 ± 9·5	226·4 ± 25·0
X		21	7	Op.	6	55 ± 5	3·8 ± 0·3	7·4 ± 0·6	14·7 ± 1·2	67·1 ± 2·7	132·9 ± 11·9	266·2 ± 12·6
				C.	6	52 ± 4	3·3 ± 0·1	7·1 ± 0·6	13·1 ± 1·0	63·7 ± 4·7	131·8 ± 13·0	250·7 ± 19·2
XI		21	64	Op.	6	200 ± 26	11·2 ± 1·0	16·9 ± 2·0	47·2 ± 5·0	56·7 ± 3·4	85·2 ± 8·5	239·0 ± 21·1
XII	A.	104–188	7	Op.–O.*	7	184 ± 11	11·4 ± 1·5	16·9 ± 2·5	48·2 ± 4·5	62·0 ± 7·7	91·2 ± 10·4	263·5 ± 24·8
	B.	128–208	7	Op.–Di.†	6	185 ± 11	10·3 ± 0·8	16·5 ± 2·1	52·2 ± 4·9	55·5 ± 3·4	88·7 ± 9·7	282·0 ± 23·5
	C.	87–207	7	Op.	17	179 ± 12	10·4 ± 1·3	17·0 ± 2·3	48·5 ± 4·8	57·8 ± 5·9	94·9 ± 12·3	271·0 ± 23·8
XIII	A.	139–155	21	Op.	5	256 ± 22	12·3 ± 0·5	21·2 ± 1·4	56·7 ± 3·5	54·3 ± 4·5	92·5 ± 2·4	248·7 ± 18·0
	B.	139–148	21	Op.	4	228 ± 8	13·5 ± 1·0	18·0 ± 1·1	39·7 ± 4·7	59·0 ± 3·7	79·1 ± 7·1	173·8 ± 19·2
XIV		80	42	Op.	6	198 ± 7	11·2 ± 0·5	17·5 ± 2·0	34·5 ± 1·9	56·8 ± 3·1	88·5 ± 11·1	174·6 ± 10·0
XV		124–142	56	Op.	4	221 ± 4	14·6 ± 0·9	15·6 ± 0·4	36·0 ± 2·1	65·9 ± 4·7	71·7 ± 3·2	161·1 ± 10·2
Controls		Mean age 128 days			23	188	12·3 ± 1·3	—	—	65·1 ± 1·9	—	—
Œstrus		,, 119 ,,			34	177	—	19·0 ± 1·6	—	—	114·0 ± 1·3	—
		,, 100 ,,			19	151	—	—	42 ±4	—	—	283 ±28
Controls		Mean age 102 days			8	170	9·1 ± 0·9	—	—	53·6 ± 4·3	—	—
Dicestrus		,, 102 ,,			7	170	—	18·2 ± 1·6	—	—	107·0 ± 4·5	—
		,, 107 ,,			29	152	—	—	36 ±5	—	—	233 ±22

* Op.–O. = operation during œstrus. † Op.–Di. = operation during dicestrus.

Data for mature controls from Andersen and Kennedy [1932] and Andersen [1933].

killed 50 days later, and under these conditions the adrenals were not hypertrophied. The difference in these findings might be due either to the difference in age at operation or in the post-operative interval. The subsequent experiments were therefore planned with the idea of obtaining accurate information as to the immediate and late effects of gonadectomy on immature and mature rats of both sexes. The rats were of the same strain as those used in our previous experiments. They were bred in the laboratory in litters limited to six at the age of 1 day. Those weighing less than 30 g. at weaning at 28 days were discarded. The majority weighed 40–65 g. at 28 days. As far as possible the rats of each litter were paired by sex and weight, and one of each pair was used for operation, the other as the control in the same experiment. The diet was that used in our previous experiments [Andersen, 1932 *a*] and was a McCollum B diet with the addition of 5 p.c. of dried yeast and a ration of fresh lettuce twice a week. Previous experiments have shown that healthy male rats of our colony mature before the seventieth day [Andersen, 1932 *b*]. The immature animals were gonadectomized at 21 or 22 days and the mature ones at 80–81 days. In a few groups the mature animals were several weeks older than this. In addition to the normal controls there were also vasectomized controls for the males. The vasectomy was performed by a double ligation with excision of 3–5 mm. of the vas between the ligatures and was always bilateral. In castration the entire testes and epididymes were removed; in the males castrated after maturity the epididymes were examined for the presence of mature motile sperm, which were found in every case. The peritoneal route was used in the operations on the 21-day-old male rats, while in the 80-day-old males the operation was performed through the scrotum. Daily vaginal smears were made on all the older female rats and only those having regular cycles were used. Rats operated on at 80 days were not previously smeared. The ovariectomy was done by the dorsal route, through small bilateral incisions in the paravertebral region. The ovary, tube, adjacent fat, and a small portion of the uterus were removed, and the remaining vessels and the cut end of the uterus were included in a single ligature. The operations were performed under ether anæsthesia and with aseptic technique. At autopsy the operative wound was examined for infection and this was present only in several of the mature vasectomized and castrated animals. These rats were discarded from the series and replaced by uninfected ones. In one series of mature females, half of the animals were spayed during œstrus and half during dicestrus. The animals of this group were somewhat older than the others, the age varying from 100 to 200 days

at the time of operation. Most of the experiments were performed during the late spring and early summer.

The animals gonadectomized at 21 days were sacrificed 1, 3, or 9 weeks after operation, and the mature groups were sacrificed 1, 3, 6, or 8 weeks after operation. The animals were killed with chloroform, and the thyroid, adrenal, and pituitary were quickly dissected out, placed in a closed weighing bottle, and weighed to 0·1 mg. These organs were then fixed in Zenker's fluid or formalin. Fresh smears were obtained from both epididymes of all vasectomized and control males. These were suspended in saline and studied for the number, motility, and maturity of the sperm.

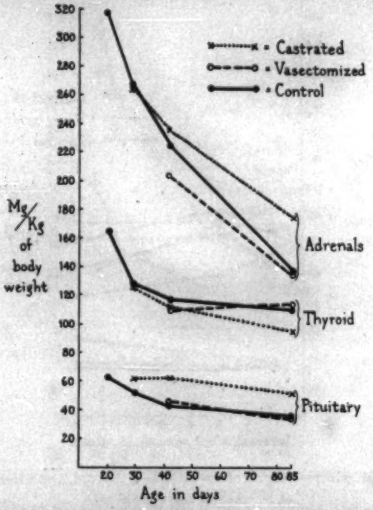
The actual weight of the organs in question and the relative weight in terms of mg./kg. of body weight were obtained for each animal and the mean of the actual and also of the relative weights of each organ were calculated for each group. The probable error of the mean was calculated by the formula used in our previous experiments [Andersen and Kennedy, 1932].

The following organs were sectioned and stained with hæmatoxylin and eosin: in the castrated males, the thyroid and adrenal; in the vasectomized males, the testes; in control males, the thyroid, adrenal, and testes; in the spayed and control females, the thyroid and adrenal. The adrenals were sectioned in three males vasectomized at 80 days and killed after 1 week. In the thyroid and testes a few sections were obtained through the thickest portion of the organ. In some animals of each group the adrenals were fixed in Zenker's and serially sectioned; every tenth section was mounted and then stained with hæmatoxylin and eosin. In some instances, as reported below, tracings were made of these sections and the volume of the cortex and medulla measured with a planimeter. In other animals of each group, the adrenals were fixed in formalin, and frozen sections were made and stained for fat with Scharlach R. The pituitaries are yet to be studied histologically and will be reported later.

(3) *The adrenal weight after gonadectomy.*

(1) *Male rats castrated and vasectomized before maturity.* In all, forty-six rats were used in this portion of the experiment. Four of these were controls killed at the age of 20–21 days to obtain the endocrine weights at the time of operation. Six castrated and six normal animals were killed at the age of 28 days (Table I, Exp. III). Four each of castrated, vasectomized, and control animals were killed at the age of 42 days (Table I, Exp. IV). No significant differences were found in the adrenal weights of the castrated animals as compared with their controls in either

of these experiments. Neither control nor vasectomized animals had mature motile sperm in the epididymes at 28 or 42 days of age. There were mature motile sperm in the epididymes in all control and vasectomized animals at 85 days. The castrated animals had significantly larger adrenals than the control animals, while the adrenals of vasectomized rats were similar to those of the controls (Table I, Exp. V). The figures for the relative weight of the adrenals of normal animals show a sharp decline

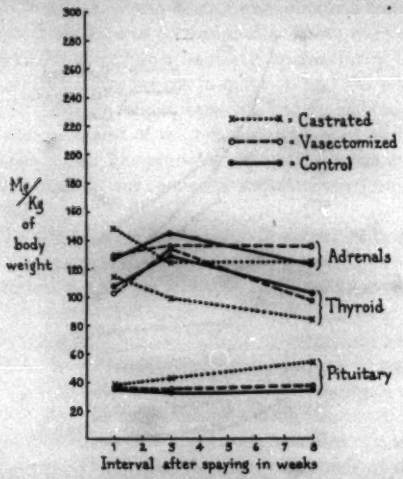


Text-fig. 1. The relative weights of the pituitary, thyroid, and adrenals after castration or vasectomy at the age of 21 days—male rats.

with age, while in castrated animals there is a marked lag in this decrease (Text-fig. 1). These figures may be supplemented by the data of Hatai [1915]: in rats castrated at 16–22 days and killed after 100–173 days he found weights similar to those reported here, showing that even after this long interval the glands do not come down to the size of those of normal animals.

(2) *Male rats castrated and vasectomized after maturity.* Forty-three rats, some of which were probably not mature, were used in the preliminary experiments, and forty-eight were used in the more carefully

standardized experiments. The latter were castrated at 80–82 days and killed after 1, 3, and 8 weeks. In all cases the epididymes of the vasectomized and control animals contained mature motile sperm and the weights of the testes were similar, showing that 8 weeks is not long enough after vasectomy for any significant atrophy of the testis to take place. The organ weights of these two groups were parallel in each case.

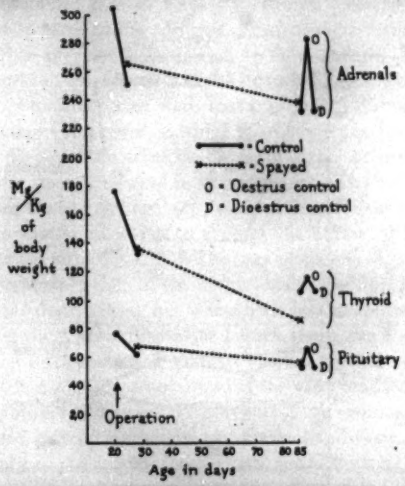


Text-fig. 2. The mean relative weights of the pituitary, thyroid, and adrenals after castration at 80 days of age—male rats.

One week after castration the adrenals were slightly enlarged (Table I, Exp. VI). A much longer series would be necessary to ascertain whether this enlargement is significant, but it does not appear so in the present series. After 3 and 8 weeks the relative adrenal weights were similar to those of the controls (Text-fig. 2). The adrenal hypertrophy after castration found in the first preliminary experiment may be attributed to the probable immaturity of some of the animals.

(3) *Female rats spayed before maturity.* This series included twelve operated and ten control animals (Table II, Exps. X, XI). Four of the controls were killed at the age of 20–21 days to show the conditions at the time of operation. In the animals which were spayed at 21 days and killed

after 1 week no adrenal changes were found as compared with the controls. Since the female rats of our colony are mature after 50 days, at 85 days they all show cyclic changes in the adrenals. The figures obtained from our previous work on adrenal changes associated with œstrus are therefore utilized as control data for the spayed animals killed at 80 days [Andersen and Kennedy, 1932]. These control rats were young mature animals not classified accurately as to body weight, but all had regular cycles (Table II).



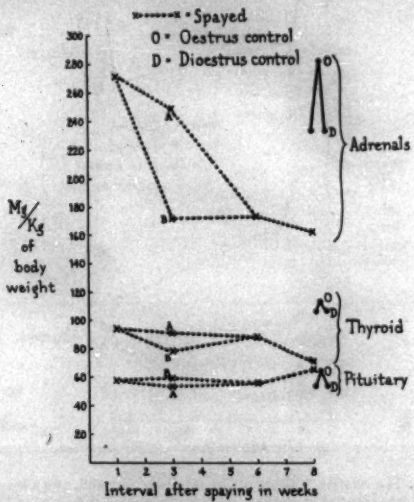
Text-fig. 3. The relative weights of the pituitary, thyroid, and adrenals after spaying at the age of 21 days—female rats.

In the spayed rats killed at 85 days, the vagina was found open and the uterus was immature in each case. No gross ovarian tissue was found. The mean adrenal weight of these rats is about the same as that in the controls in dioestrus (Table II, Exp. XI; Text-fig. 3).

(4) *Female rats spayed after maturity.* Twenty-one rats were used in the preliminary experiment, of which twelve were spayed and eleven were controls (Table II, Exp. IX). They were spayed between the ages of 53 and 73 days and killed after 1–2 months. Thirty-six spayed animals were included in the later series. The control data was obtained from our previous work on œstrus changes [Andersen and Kennedy, 1932]. The

operation was performed between the ages of 80 and 200 days, and the animals were killed after intervals of 1, 3, 6, and 8 weeks.

Seventeen animals were killed 1 week after spaying. The adrenal was hypertrophied with a mean relative weight similar to that found at œstrus and well above that found at dioestrus. Seven of the animals were spayed at œstrus and six at dioestrus, while in four the phase of the cycle was not noted. No difference was found in the mean adrenal weight of these three groups (Table II, Exp. XII; Text-fig. 4).



Text-fig. 4. The mean relative weights of the pituitary, thyroid, and adrenals after spaying at the age of 80–200 days of age—female rats.

The animals killed 3 weeks after operation were in two groups, of four and five rats respectively (Table II, Exp. XIII). Group A was killed in the autumn and Group B was killed in the early summer. The adrenals in Group B were atrophied to a degree similar to that found in animals killed after a longer interval. It seems probable that some factor, probably seasonal, delayed the progress of the usual changes.

In the rats killed 6 and 8 weeks after operation the relative adrenal weight was about 30 p.c. below that found at œstrus and only slightly

above that found in young mature males (Table II, Exps. XIV, XV). These figures corroborate those obtained in the preliminary experiment (Table II, Exp. IX).

(4) *Histological observations.*

Serial sections were made of both adrenals of two or three of the castrated male rats and of an equal number of controls of Exps. III, V, VI, VII, and VIII (Table I). Serial sections were also made of the adrenals of three vasectomized rats of Exp. VI. Frozen sections stained with Scharlach R were made of the adrenals of two or more castrated rats and an equal number of controls from each of these groups. No differences were found between the adrenals of the operated and control animals of Group III, in which the rats were castrated at 21 days and killed at 28 days. In Group V, however, in which the rats were castrated at 21 days and killed at 85 days, the adrenals of the operated animals retained to some extent the structure found in immature animals (Pl. I). The cortex was wider than that of the controls, and much less clearly divided into zones. It was similar in width to that of the normal dicestrus female [Andersen and Kennedy, 1932]. The zona glomerulosa was narrow and less clearly demarcated than in the normal male. The lipoid had approximately the same distribution, but was a little less abundant. In the rats castrated at 80 days and killed after 1 week there was a slight increase of lipoid in the fasciculata and marked engorgement in the inner third of the cortex (Pl. I). This was not found in the vasectomized animals. No difference was observed between the adrenals of the animals of Groups VII and VIII, killed 3 and 8 weeks after castration, and their controls.

In the study of the female spayed rats, serial sections were made of both adrenals of at least two control and two operated rats in each of Exps. X, XI, XII, and XIV (Table II), and fat stains were done on the adrenals of at least two rats of Exps. XI, XII, and XIV. The animals used in our previous studies on the cestrous cycle [Andersen and Kennedy, 1932; Andersen, 1933] served as controls for the mature animals, while controls of the same age were studied in the case of immature animals.

The adrenals of animals killed at the age of 28 days were all similar regardless of sex or gonadectomy. In the female rats spayed at 21 days and killed at 85 days of age the fasciculata contained a moderate amount of lipoid, but not as much as is present in animals at cestrus. The groups of cells near the medulla were not prominent and the glomerular zone was sharply demarcated in some cases and not in others. The glands resembled

those of the castrated males in the corresponding experiments. In the animals spayed at 80 days and killed after 1 week, the adrenals were indistinguishable from those found at œstrus in normal animals (Pl. II). This was true regardless of whether the operation was performed at œstrus or diœstrus. The engorgement of the inner third of the cortex was marked. In those of Group XIV, killed 6 weeks after spaying, the cortex was greatly narrowed, with a sharply demarcated glomerulosa, a moderate amount of lipoid in the outer half of the fasciculata. The more striking changes are those in the inner half of the cortex, where the capillaries show extreme congestion with narrowing of the cell columns, decrease in size of the cells, pyknosis of most of the nuclei, and a deeper eosinophilic stain in the cytoplasm (Pl. II).

In summary, we may say that the adrenals of animals spayed before maturity resemble those of their controls of the same age until the latter have become mature. The adrenals of the operated animals then retain in part the structure of those of the immature animals. In the case of gonadectomy after maturity there is a temporary increase in lipoid in the fasciculata, more marked in the females, with congestion in the inner third of the cortex. In the male 3 weeks after castration the adrenals appear similar to those in normal animals. The process of regression is slower in the female, with degenerative changes in the inner portion of the cortex and marked congestion of the capillaries.

(5) *The volume of the cortex and medulla.*

The relative volumes of the cortex and medulla of two male castrated rats and their normal controls from Exp. III and an equal number from Exp. IV have been measured and calculated by the same method used in our previous work [Andersen and Kennedy, 1932].

Similar studies have been made on two female rats of Exp. XII. This was done by making tracings of every tenth section (cut at 5 μ), measuring the area of the adrenal and of the medulla with a planimeter and calculating the percentage of the total volume occupied by the medulla. On the assumption that the specific gravity of the cortex and medulla is approximately the same, the relative weight of each was calculated. The results are given in Table III. The series is too small to give more than an indication of the volume changes. It is obvious, however, that the medulla is about equal in size in the four adult males of the series, and slightly larger in the two females in the series, as well as in the female controls. This sex difference may only be apparent as the greater lipoid content of the female cortex would make for greater shrinkage in the process of

Fig. 1. Adrenal of a normal male rat, aged 87 days. H. and E. × 50.

Fig. 2. Adrenal of a male rat which was castrated at 21 days and killed at 85 days. H. and E. × 50.

Fig. 3. Adrenal of a male rat which was castrated at 80 days and killed after 1 week. H. and E. × 50.

Fig. 4. Adrenal of a female rat which was spayed at 80 days and killed after 1 week. H. and E. × 50.

Fig. 5. Adrenal of a female rat which was spayed at 80 days and killed after 6 weeks. H. and E. × 50.

Fig. 6. Central portion of the thyroid of a normal rat aged 136 days. H. and E. × 217.

Fig. 7. Central portion of the thyroid of a male rat castrated at 80 days and killed at 136 days. H. and E. × 217.

preparing the sections, and the medulla would therefore appear slightly larger. The change in size in the gonadectomized animals is mostly if not entirely due to change in size of the cortex.

TABLE III. The weight of the adrenal cortex and medulla of the rat.

Experiment	Rat No.	Weight in g.	Adrenals		Cortex	Medulla
			mg.	mg./kg.	mg./kg.	mg./kg.
Males castrated at 21 days, killed at 85 days	1149 Op.	251	51·0	203·0	190·6	12·4
	1147 Op.	199	34·1	171·2	159·1	12·1
Male controls aged 85 days	1151 C.	196	24·3	123·9	112·4	11·5
	1150 C.	215	25·1	116·8	104·4	12·4
Females spayed at 80 days, killed at 87 days	1413 Op.	182	41·0	225·0	207·4	17·6
	1415 Op.	156	47·9	307·0	285·2	21·8
Female controls aged 61–73 days:						
Œstrus	Av. of 5	149	40·9	278·4	257·3	16·9
Dioestrus	Av. of 5	147	35·0	238·8	221·0	17·9

Data on female controls from Andersen and Kennedy [1932].

II. THE THYROID.

(1) *Review of literature.*

So far as could be ascertained the earliest observations as to the effect of castration on the thyroid are those of Cecca [1904] and of Tescione [1904], who concluded that the operation was followed by a temporary hypertrophy and later by atrophy. Martini [1904] reported changes which he interpreted as those of hypersecretion. These papers are not available to us, and the species investigated are therefore not known. Most of the subsequent work has been done on rodents.

(1) *Rabbit.* Soli [1909] reported hypertrophy of the thyroid after gonadectomy followed by decrease in the size of the gland after about 3 months in seven male and three female gonadectomized rabbits, as compared with an equal number of controls of the same age and sex. Engelhorn [1912], in a paper which we have been unable to obtain, reported hyperplasia with increase of colloid and flattening of the epithelium in the thyroids of female rabbits 3 months after spaying. Bell [1913] recorded similar findings in castrated rabbits and interpreted them as evidence of increased thyroid activity. Livingston [1916], in a long series of rabbits which, however, showed extreme variations in age, weight, and post-operative interval, concluded that the thyroid atrophied slightly in the male, but showed no change in weight after gonadectomy in the female. He gave a detailed report of his experiments, and inspection of the thyroid weights reveals such wide variations as to make the

difference between the mean weights of the castrated and control animals insignificant. Okuneff [1922] in seventeen rabbits killed 2–5 months after castration found an increase in colloid in the follicles with a decrease of lipoid in the epithelial cells, which he interpreted as evidence of increased thyroid secretion. Saito [1930], giving no details of his experiments, reported decrease of thyroid weight in both sexes with an increase in iodine content. In the rabbit, therefore, no adequately controlled studies have been reported in sufficient detail to establish the presence or direction of a change in weight of the thyroid after gonadectomy. Engelhorn, Bell, and Okuneff are unanimous in stating that there is an increase in colloid in the follicles, and the two former workers agree that the epithelium is flattened.

(2) *Guinea-pig.* No observations on the weight of the guinea-pig thyroid after castration have been found. Constantini [1929] found no constant change in the histological picture 1 month after castration. Chouke [1930] confirmed this and also reported no change in the number of mitoses in fourteen female guinea-pigs, killed at intervals of 14–323 days after operation, as compared with six controls, none of which was in heat. There was an increase in the three controls killed during heat.

(3) *Rat.* The first and most careful observations on weight changes in the rat thyroid are those of Hatai [1915]. The plan of the experiments has been given in the above discussion of the observations on the adrenals. The male rats castrated before the twenty-second day and killed 3–5 months later had marked thyroid atrophy, but in the females spayed before maturity the mean thyroid weight showed an insignificant increase in weight. In the male rats castrated after maturity there was a slight thyroid atrophy. The females spayed after maturity varied in their response: those spayed at 97–119 days and killed after 98–100 days had marked atrophy, while those castrated at 172–195 days and killed after 27–60 days showed a slight increase in the weight of the thyroid. Inspection of the figures shows that this discrepancy is due to a change in the controls. The mean thyroid weight is approximately the same for the two groups spayed after maturity, but the weight of the thyroids in the older control group is very much less than in the younger control group and is comparable to that found in our older anœstrous animals (data unpublished).

Matsuyama [1921] gave no weights, but observed histological changes in rats killed several months after castration. These consisted of flattening of the epithelium with an increase of colloid in the follicles, which stained more deeply with eosin. Furuya's observations [1924] on

the altered compensatory hypertrophy in spayed rats after removal of one lobe of the thyroid are based on few animals and also on the false assumption that the two lobes of the thyroid are of equal weight. It can be disregarded. Minowada [1928] with twelve castrated and five control male rats observed a decrease in the size of the follicles in the former. His figures show a slight decrease in the mean adrenal weight of the gland. Bokelmann and Scheringer [1932] found some atrophy in eleven adult females killed 5 weeks after operation as compared with an equal number of controls, and obtained similar results in a somewhat larger number of animals killed 6 months after operation. Calculations based on their figures show that the difference between the mean and control thyroid weights is not significant because of the wide variations. They analysed the glands for iodine and reported an increase, which also does not bear statistical analysis, but which confirms the findings of Saito in rabbits.

We are left with the impression that castration produces atrophy of the thyroid in the rat, but the variation of the weights in the protocols of Bokelmann and Scheringer, and the hypertrophy reported by Hatai in the females spayed before maturity and those spayed after the age of 6 months leaves room for doubt. The histological report of Matsuyama does not regard the differences of age, sex, and post-operative interval which, according to Hatai, are so significant.

(4) *Other species.* Bell [1913] found no change in the thyroid of the cat after gonadectomy. No investigations on dogs have been found. Merschejewsky in Korentschewsky's laboratory is reported by the latter [1914] to have found atrophy of the thyroid in castrated oxen and horses. Okuneff [1922] observed an increase of colloid in the follicles, and a decrease of lipoid in the epithelial cells of the thyroid of oxen. No constant change was found by Soli [1909] in capons. Atrophy of the thyroids was reported in eunuchs by Tandler and Gross [1913].

In all these experiments it is obvious that only Hatai investigated the effect of castration in relation to the age at operation, and only Chouke has noted whether the female controls were killed during œstrus, or whether their reproductive activity was normal.

(2) *The thyroid weight.*

The data were obtained from the animals used for the observations on the adrenals, except in the case of mature female controls, for which the previous report on œstrous changes was utilized [Andersen, 1933]. In all cases the vasectomized rats resembled the controls.

(1) *Male rats castrated before maturity.* There was no significant difference between the mean relative thyroid weights of the castrated and control rats killed at 28 and 42 days. At 85 days there was a slight but definite decrease in the thyroid weight of the castrated rats (Text-fig. 1).

(2) *Male rats castrated after maturity.* No change in thyroid weight was observed after 1 week. After 3 and 8 weeks a moderate atrophy was noted, which was not statistically significant (Text-fig. 2).

(3) *Female rats spayed before maturity.* No change was found at 28 days. However, at 85 days the mean adrenal weight of the thyroid was definitely below normal (Text-fig. 3).

(4) *Female rats spayed after maturity.* The thyroid showed some atrophy in each series. This was significant after 1 week, and after 3 weeks was greater in Group B than in Group A. After 8 weeks the mean relative thyroid weight was about 25 p.c. below that found in normal animals (Table II, Exp. XI; Text-fig. 4).

(3) *Histological observations on the thyroid.*

Sections were made of the thyroids from four male rats castrated at the age of 21 days and killed at the age of 28 days; from four male rats castrated at the age of 21 days and killed at the age of 85 days; and from an equal number of male controls for each group. No difference was found at 28 days between the thyroids of the operated and those of the control animals. However, in those killed at 85 days a distinct difference was noted. In all rats the peripheral follicles are larger than the central ones and have more colloid and a lower epithelium. In the castrated animals the thyroid is definitely smaller, and the irregularity of the decrease in weight is at least partly explained by the presence of various amounts of fat and muscle removed and weighed with the glands in almost every instance. In the castrated rats the follicles are on the whole smaller than the follicles in corresponding portions (*i.e.* periphery or centre) of the control glands. In the central portion the epithelium is lower, with a darker, less abundant cytoplasm and more distinct nuclei which lie in about the centre of the cells. The epithelial border is more sharply demarcated. The colloid appears denser, is more eosinophilic, contains fewer vacuoles and includes fewer desquamated cells (Text-fig. 6). These changes are found to a lesser degree in the peripheral vacuoles. In the controls many of the thyroid follicles contain hyperchromatic nuclei, apparently originating from desquamated epithelial cells. Some of the nuclei of the epithelial cells are also small and hyperchromatic. The colloid, by comparison with the operated animals, is pale, even in consistence, and contains an abundance

of vacuoles. The blood vessels and capillaries contain more blood and are therefore more conspicuous.

In thyroids of four male rats castrated at 80 days and killed after 1 week, a suggestion of similar changes is found as compared with the four controls of the same age. These changes are not sufficiently striking so that the diagnosis of castrated as against control animals can be made from inspection of the thyroid. The most definite change is in the colloid, which appears denser, more eosinophilic, and has a much less foamy appearance. In the four males castrated at 80 days and killed after 8 weeks the thyroid changes, as compared with the controls of the same age, are the most marked of those found in any group. They resemble those found in the animals castrated at 21 days and killed at 85 days. The decrease in the size of the follicles is obvious. The epithelium is flattened so that the nucleus occupies most of the cell volume and there appears to be only cell membrane between the nucleus and the lumen of the follicle. In the central follicles the nucleus remains round, but in the peripheral follicles it is flattened and hyperchromatic, and in some places the cells are several times as wide as they are thick in the radial axis of the follicle. Blood vessels are less conspicuous than in the normal thyroids.

We may therefore say that there is a definite change in the histological picture of the male thyroid after castration, whether the operation is performed before or after maturity, and that this change is clear-cut in rats killed 8 weeks after castration.

The observations on the female thyroid after spaying are based on sections of both lobes from four animals which were spayed at the age of 80 days and killed after 6 weeks, at which time the thyroid shows a definite decrease in weight. For a control, sections were made of both lobes from nine normal rats which had had regular cycles of 5 days or less; four of these were killed at œstrus and five at diœstrus. In eight of these rats the histological picture was similar, and resembled that found in the normal males of the same age. The impression was that on the whole the colloid was a little more vacuolated and the cells a little larger and more clearly outlined than in the males, but it was impossible to make an accurate diagnosis of male thyroid as against female thyroid by inspection of the sections. The ninth thyroid, that of a rat at diœstrus, somewhat resembled those of the spayed animals. The thyroids of the spayed animals were similar to those found in the male rats castrated at 80 days and killed after 8 weeks. It was therefore possible to recognize a thyroid from inspection of the sections as that of a normal or gonadectomized animal, but it was not possible to state the sex in either case.

III. The pituitary.

(1) *Literature.*

The literature on the effect of castration on the pituitary has been recently reviewed by Schenk [1927] and by Severinghaus, Engle and Smith [1932]. Most authors agree that there is hypertrophy, but the histological changes are still a matter of dispute. As we have as yet made no adequate histological studies on the pituitary, the literature will not be reviewed here.

(2) *The weight of the pituitary.*

The data were obtained from the animals used for the adrenal studies, except for the mature female controls, which have been previously reported [Andersen, 1933]. In all cases the vasectomized rats resembled the controls.

(1) *Male rats castrated before maturity.* At 28 days the mean relative weight of the pituitary in the castrates was greater than that of the controls, but the probable error was sufficient so that the difference was not significant. At 42 days, however, the pituitary was definitely larger. Calculation shows the relative weight of the pituitary to be the same in 21-day controls, and in rats castrated at 21 days and killed at 28 or 42 days, while in the normal animals the relative weight of the pituitary decreases with age. In the castrated animals killed at 85 days the relative weight of the pituitary is less than in the 42-day-old castrated rats, but is about 42 p.c. above that of the controls (Table I; Text-fig. 1).

(2) *Male rats castrated after maturity.* The relative weight of the pituitary does not increase much during the first week after castration, but is about 26 p.c. greater than in the controls after 3 weeks. By the eighth week it is about 57 p.c. greater. The pituitary, therefore, hypertrophies gradually after castration throughout the 8-week period studied (Table I; Text-fig. 2).

(3) *Female rats spayed before maturity.* No change was found in rats killed at 28 days. At 85 days the mean relative pituitary weight of the spayed animals lies between that of the controls in œstrus and of those in diœstrus (Table II, Exp. XI; Text-fig. 3).

(4) *Female rats spayed after maturity.* After 1 week the pituitary was significantly larger in the rats which were spayed during œstrus than in those spayed during diœstrus (Table II, Exp. XII). In the two groups of rats killed after 3 weeks the mean relative pituitary weight was greater in Group B, which also showed more advanced adrenal and thyroid

changes, but lay between the mean relative pituitary weight in normal rats at œstrus and dicestrus in both groups. After 8 weeks the mean relative pituitary weight had increased to but not above the normal œstrous weight. There is, therefore, no hypertrophy of the pituitary after spaying in the mature female rat (Table II; Text-fig. 4).

IV. Histological observations on the testes of vasectomized animals.

Sections have been made through the thickest portions of one testis from each of four vasectomized and four normal control rats of each group in which vasectomized controls were used. In no case do the testes of vasectomized rats differ from those of the controls in the histological appearance of either tubules or interstitial cells. Vasectomy, whether performed before or after maturity, therefore, does not affect spermatogenesis in the period of eight weeks. No abnormalities were found in the approximate quantity, appearance, or motility of sperm obtained from fresh smears of the epididymis.

V. Discussion.

It is obvious from inspection of the data in Tables I and II that the effect of castration on the adrenals in the rat varies not only with the sex and with the age at operation, as pointed out by Hatai [1915], but with the interval after operation. These experiments have been planned in consideration of these points, and with the advantage of knowledge of the cyclic changes in the female adrenal. As in the case of œstrous hypertrophy, changes have been found only in the cortex. In normal rats 21 days of age, the relative adrenal weight is the same in both sexes. At 85 days the relative adrenal weight is more than twice as great in the normal female rat as in the normal male. In animals of both sexes gonadectomized at 21 days and killed at 85 days, the relative adrenal weights lie between those of the male and female controls. They are somewhat greater in the spayed female than in the castrated male. It is curious that removal of the gonads in immature animals, at an age when the adrenals show no sex differences, should not permanently remove sex differences in that organ. It is useless at present to speculate as to whether this is due to a primary sex difference in some other organ, perhaps the hypophysis, or whether the presence of the gonads during the first 3 weeks of life has permanently affected the endocrine glands, even though these

organs were not functioning in a reproductive capacity, and had not at that age produced any discernible sex differences in the pituitary or adrenal.

A similar problem confronts us in regard to the effects of the operation on the mature animals. In these mature rats, however, another interesting question is brought up by the temporary increase in cortical lipoid, more marked in the females. The effect of an operation *per se* has been ruled out in rats killed 1 week after operation because of the absence of this change after vasectomy. This hypertrophy is followed by return to normal in the male and by degenerative changes in the female. By 6 weeks, however, the female adrenals have reached their final low weight in our series. The changes in the spayed female may be partly due to the absence of œstrus. The fact that the decrease in weight is greater in spayed animals than in animals in diœstrus may be interpreted either as meaning that there is an adrenal-stimulating hormone present during diœstrus as well as œstrus, or that the regressive changes in the adrenal after œstrus are not completed before the arrival of the next cycle in the rat. If the latter were true one would expect the adrenals to be the same in spayed animals and in animals in diœstrus of species having long periods between œstrus. This would explain the negative findings of some workers in the dog, rabbit, and guinea-pig. The confusion in the literature in regard to adrenal changes after castration may be explained by the usual disregard of the presence of infection, and the frequent disregard of other factors such as age and weight at operation and autopsy, the presence or absence of œstrus in the female controls, the uniformity of diet, breed, and post-operative interval. The question may be considered unsolved in the rabbit and dog. There is probably a transitory hypertrophy in mature male guinea-pigs, with a normal adrenal weight in both sexes some weeks after operation. The effect in the rat and mouse has been shown to vary with the sex of the animal and the age at operation. The presence of the x zone in male mice castrated before maturity is comparable to the finding of adrenals resembling those of immature animals in our similar experiments on rats.

Gonadectomy produces atrophy of the thyroid of the rat in both sexes and regardless of the age of operation. It is not definitely established after 3 weeks, but is clear after 8 weeks. It is most marked in the mature female 8 weeks after spaying. The impression obtained on reviewing the literature on the subject is that gonadectomy produces thyroid atrophy in most of the species investigated, although much of the work is open to criticism.

The hypertrophy of the pituitary after gonadectomy is well known, and the only contribution to the problem which is presented here consists of specific data on the sex, age, and time intervals involved. In rats of both sexes gonadectomized at 21 days and killed at 85 days, the pituitary weighs approximately 53–58 mg. per kg., which is greater than that of the male controls and about the same as the female controls at dicestrus.

In the mature male rat the pituitary hypertrophy is gradual and continues throughout the 8-week interval studied. In the female there is but little change in the weight of the pituitary in 1 week. Those spayed during œstrus have heavier glands than those spayed during dicestrus, but both are within the weight range found at dicestrus. At 3 and 6 weeks the gland is slightly larger than at dicestrus, while after 8 weeks it has regained the œstrous weight. Therefore there is no increase in the pituitary weight of the spayed female over the normal range of weight. At 8 weeks after operation the pituitary is larger in the female than in the male of the corresponding experiment.

It is interesting to observe that the changes in the thyroid, adrenal, and pituitary of gonadectomized animals as compared with controls, are not established until the controls are mature, in the case of animals gonadectomized at 21 days; and they are not established at 3 weeks and are established at 8 weeks after operation in the case of the animals gonadectomized after maturity. The various changes, therefore, require approximately the same time and are undoubtedly related.

The interpretation of the significance of these changes in the endocrine glands after castration cannot yet be made. Recent studies on the relation of reproductive changes to metabolism such as those of Okey and Boyden [1927] on the cyclic changes in blood lipoids in women and those of Kaufmann and Mühlbock [1931] on changes in the lipoid metabolism after ovariectomy in women indicate a probable source of enlightenment on the question.

The normal number, appearance, and motility of spermatozoa up to 8 weeks after vasectomy, together with the normal weights of the endocrine glands and normal histological appearance in these animals is an indication that the operation in itself has no effect and that vasectomy is in no way comparable to castration in a physiological sense, at least for the first 8 weeks.

The fact that the vagina opened before the age of 85 days in all of our animals spayed at 21 days is proof that the opening of the vagina is not dependent entirely on coincident ovarian activity. The uterus was immature and no ovarian tissue was found in these animals.

VI. Conclusions.

I. The effect of gonadectomy on the adrenals of the rat varies with the sex of the animal, the interval after operation and the age at operation. Measurements of the volume of the cortex and medulla in a small group of rats show that effects of castration are evident only in the cortex, the medulla remaining approximately uniform in volume and showing no histological changes. The findings in the various experiments are as follows:

(1) In male rats castrated at the age of 21 days and killed 1 or 3 weeks after operation, the adrenal shows no changes in weight or histological appearance as compared with the controls. Nine weeks after operation the adrenals of the castrated animals are hypertrophied, contain more lipoid than the controls, and histologically resemble the adrenals of immature animals.

(2) In female rats spayed at the age of 21 days and killed after 1 week the adrenals resemble those of the controls of the same age. When killed after 9 weeks the adrenals have approximately the same relative weight as those of controls in dicestrus and weigh more than those of the males of the corresponding experiment, but resemble them histologically.

(3) In male rats castrated after maturity and killed after 7, 21, or 56 days there is no significant change in the adrenal weight. One week after operation there are histological changes consisting of increased lipoid in the fasciculata and congestion of the reticularis. The histological appearance returns to normal.

(4) In female rats spayed after maturity and killed after 1 week the adrenal is hypertrophied and has the histological appearance of the adrenal at œstrus. This is true whether the rat was in œstrus or in dicestrus at the time of operation. In one group of rats killed after 3 weeks the adrenals showed marked atrophy, while in a second group they did not. The adrenals of rats killed 6 or 8 weeks after spaying are all atrophied, with decrease of lipoid in the fasciculata and degenerative changes and engorgement in the reticularis.

II. No changes are found in the thyroid 1 week after castration, but there is atrophy after 3 weeks in some experiments. A definite atrophy is apparent 8 weeks after castration in all cases, which is accompanied by characteristic histological changes.

III. There is hypertrophy of the pituitary after castration of either immature or mature male rats, which is progressive throughout the 8-week interval studied. In the spayed female rats the pituitary weight does not

become greater than that found at œstrus, but is somewhat greater than in the castrated male rats.

IV. A critical survey has been made of the literature on the effect of gonadectomy on the adrenal in various species. No constant change has been definitely proven in the rabbit, dog, ox, sheep, and capon. There is a temporary hypertrophy after spaying in the adult female guinea-pig, and a hypertrophy was found 2 weeks after gonadectomy in the frog. Changes in the rat and mouse vary with the sex and age at operation. The literature on the thyroid is contradictory, but the balance of evidence is for atrophy after gonadectomy.

V. Vasectomy does not affect the number, appearance, or motility of the spermatozoa, nor does it affect the pituitary, thyroid, or adrenal within a period of 8 weeks after operation.

VI. Spaying before maturity delays, but does not prevent the opening of the vagina.

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DIFFUSION OF INORGANIC PHOSPHATE INTO AND OUT OF THE SKELETAL MUSCLES AND BONES OF THE FROG.

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If a skeletal muscle from a frog is immersed in a Ringer's solution containing urea, the urea distributes itself between the muscle and the solution in such a manner as to indicate that the whole of the water of the muscle is available to dissolve urea [Eggleton, 1930]. Recently it has been found that the same statement is true for the amino acid histidine [Eggleton and Eggleton, 1933], but the behaviour of the dipeptide carnosine in similar experiments indicated that only part of the water of the muscle was available to carnosine diffusing in from surrounding saline. This fraction was of the order of 30 p.c. of the total water of the muscle.

It has been shown by Stella [1928] that inorganic phosphate can be exchanged between muscles and surrounding saline solutions, and this communication presents the results of an attempt to determine what proportion of the water in the muscle is concerned in this phosphate exchange. The investigation is more difficult than was the case with urea and histidine, for in this case the muscle already contains a quantiy of the particular solute under investigation, and further, this quantity does not remain even approximately constant in an excised muscle unless special precautions are taken.

To reduce this last source of error to a minimum the experiments described in this paper have been performed at a temperature of 2° C. and, with certain stated exceptions, in an atmosphere of oxygen. The first experiments were designed to determine what, under these conditions, is the apparent concentration of phosphate (c) in the muscle when measured by the exchange of phosphate between such muscles and Ringer's solutions containing various concentrations of phosphate.

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The value of c in resting oxygenated muscles.

Two workers have already made estimates of this value. Stella [1928], working with intact hindlimbs of the frog (English and Dutch: *R. temp.* and *R. esc.*) at a low temperature, concluded that a concentration of 8 mg. P per 100 g. water in a surrounding saline prevented loss or gain of phosphate by these preparations. Semeanoff [1931], working with isolated sartorii (Hungarian *R. esc.*) at room temperature, found a value of approximately 20 mg. P per 100 g. water. The experiments recorded in Table I were performed in essentially the same manner as those of Stella and Semeanoff.

The evaluation of the amount of phosphate exchanged requires a measurement of the initial and final volume of Ringer's fluid as well as of the initial and final concentration of phosphate in it. Stella ignored the possibility of water exchange; Semeanoff states that she observed none. In the present experiments water exchange was always taken into account, and proved to be an important consideration where the concentration of phosphate in the Ringer's fluid altered only slightly. The calculation took the form:

Phosphate exchanged
= (Initial volume of Ringer's fluid × initial concentration of phosphate)
− (Final volume of Ringer's fluid × final concentration of phosphate).

In the majority of experiments described in this communication the Ringer's solutions were buffered at an initial *p*H of 8·0 (in distinction to that of 7·1–7·2 used by Semeanoff and Stella), so that they might be more comparable in this respect with phosphate-free solutions (buffered with bicarbonate). In a certain number, however (Table I, Group B 1, β; Table III, Group A 2; Table VIII, Group A 2), the Ringer's solutions were buffered at *p*H 7·1, but no difference was observed in the properties of muscle under investigation. It was noted, however, that in the conditions here used (approximately equal quantities of muscle and Ringer's fluid, in a CO_2-free atmosphere) the *p*H of the Ringer's fluid was ultimately about 7·4 for resting muscles whether the initial *p*H was 7·1 or 8·0. The quantity of saline was therefore sufficiently small for its reaction to be controlled by the muscle. In the case of fatigued muscles an initial *p*H of 7·1 in the saline was changed to 6·8.

The results quoted in Table I indicate that the immediate past history of the frog may affect the result obtained. The muscles of frogs (Hungarian *R. esc.*) which had been kept at 2° C. for only a few hours before use appeared to be in equilibrium with a phosphate concentration of about

20 mg. P per 100 g. water. If the frogs had been for 2–3 days at this temperature, the equilibrium concentration appeared to be between 10 and 15 mg. P per 100 g. water. Stella's frogs were kept "for several days" at 2° C., and his lower equilibrium value of 8 mg. per 100 g. water may be due to this difference. Semeanoff's frogs were not cooled, and her experiments were carried out at room temperature.

TABLE I. The exchange of inorganic phosphate (I.P.) between resting frog muscles and Ringer's solutions containing I.P., at 2° C.

Group	Muscle preparation	Duration of exp. hours	I.P. gained by muscle mg.	I.P. concentration in Ringer's fluid finally, mg. per 100 g. water	Equilibrium value (*c*) mg. I.P. per 100 g. water
A	Isolated muscles	3	−0·0535	7·5	
		3	−0·0147	11·4	
		22	−0·046	17·1	
		22	−0·006	20·8	About 20
		3	0·0045	19·7	
		22	0·009	30·9	
		3	0·022	29·9	
B 1 α	Isolated muscles	3	−0·0063	13·25	
		3	−0·0225	14·1	
		3	0·006	12·7	
		2½	0·0006	13·2	
β	"	3	−0·0023	11·4	10–15
		3	0·005	11·1	
		3	−0·0027	11·6	
B 2	Thigh preparation	19½	−0·0478	9·84	
		19	0·01	12·7	
		18	0·13	14·83	

The frogs used for Group A had been kept at 2° C. for a few hours only; those used for Group B had been cooled for 2–3 days.

THE PROPORTION OF THE MUSCLE CONCERNED IN THE PHOSPHATE EXCHANGE.

Having established that a Ringer's solution containing phosphate in a concentration of 8 mg. per 100 g. water is not altered by exposure to resting muscle, Stella raised the very pertinent point that "these values" might "refer to an equilibrium of phosphate between the Ringer's solution on the one hand, and the liquid, blood or lymph, on the other hand, which is present in the interspaces between the muscle fibres, and that from such experiments we get no idea of what is happening inside the muscle fibres themselves." He performed certain experiments designed to measure the proportion of the muscle concerned in the phosphate exchange. His values lay between 50 and 80 p.c. of the weight of the muscle tissue on his hindlimb preparations, and he concluded that the whole of the muscle was probably concerned in the exchange.

The experiments, the results of which are given in Table II, were designed to the same end, though they differ in two respects from Stella's. In the first place, isolated muscles were used, and in the second place the amount of phosphate gained by the muscle was measured by analysis of the muscle and not of the Ringer's fluid. The technique and interpretation were as follows.

One of a pair of muscles was killed without previous immersion, while the other was placed in a large volume (10 g. per g. of muscle) of oxygenated Ringer's fluid, containing phosphate, for a given time, and then killed. Proteins were removed by the use of trichloro-acetic acid, and the total acid-soluble phosphorus determined in each muscle [Martland and Robison, 1926]. Any increase in the total acid-soluble phosphate content was taken to be a measure of the amount of inorganic phosphate diffused into the muscle. Direct determination of the inorganic phosphate was considered less likely to yield accurate results, owing to the relatively large amount and ready lability of phosphagen present in such resting muscles. The inorganic phosphate concentration in the Ringer's fluid finally was also determined.

As in the case of Stella's experiments the interpretation of the results required some assumption as to the concentration of inorganic phosphate in that part of the muscle concerned in the phosphate exchange. On the basis of the experiments given in Table I, this was taken to be 12½ mg. per 100 g. water. The amount of water in the muscle concerned in the exchange was then obtained by dividing the amount of phosphate diffused by the difference between the final phosphate concentration in the Ringer's fluid and the assumed

TABLE II. Diffusion of inorganic phosphate (I.P.) into resting frog skeletal muscle (measured by direct analysis of the muscles).

Duration of exp. hours	T.P. content of muscle			I.P. conc. in Ringer's fluid finally	Proportion of muscle water (α) into which I.P. has diffused
	Initial	Final	Increase		
	mg. per 100 g. muscle H_2O			mg. per 100 g.	p.c.
½	157½	183½	26	126	23
1	156	188	32	126	28
2	157½	191½	34	126	30
2	162	184	22	120	20
2	158	187	29	120	27
3	171	187½	16½	126	15
4	157½	186½	29	126	25
4	164	178	14	120	13
4	179½	209½	30	120	28
5½	174½	207½	33	126	30
8	164	192½	28½	120	26
8	179½	199	19½	120	18
23	172	190½	27½	126	24
23	171½	206½	35	126	31
23	180½	202	21½	126	19
				Mean	24
				Probable error of mean	±2

For derivation of α see text.
T.P. = total acid-soluble phosphate.

value of the initial concentration in the muscle. The result is expressed in Table II as a fraction of the total water of the muscle, which is taken to be 80 p.c. of the weight of the muscle [Hill and Kupalov, 1930].

The value of this fraction, which will be called α, may be expected to increase as the period of immersion is lengthened, until the muscle is at equilibrium with the Ringer's fluid. The results in Table II show that equilibrium is attained very rapidly by the small muscles (sartorii, semitendinosi and graciles minores) used in these experiments. There is a rather large random variation, but there is no evidence of a tendency for the value of α to increase when the period of immersion is increased from 1 hour to 23 hours. Only 24 p.c. of the water of the muscle appears to be concerned in the exchange of phosphate between muscle and saline[1]. It may be remarked in passing that this value depends only slightly on the value assumed for c. If c be taken as 8 mg. per 100 g. water, α becomes 23 p.c., and if it be 20 mg. per 100 g., α has the value of 25½ p.c. But no reasonable estimate of the value of c would give values of α as high as those obtained by Stella.

It is natural to identify this fraction of the muscle water with the fluid occupying the spaces between the muscle cells, and to identify the remaining fraction, which appears to be completely enclosed by semi-permeable membranes, with the cells themselves. For the sake of brevity these fractions will be called "interspaces" and "cells" respectively, although we have no direct experimental evidence as to whether these fractions are identical with the anatomical interspaces and fibres: for histological appearances cannot be relied upon, since shrinkage of the cells during preparation of the tissue is inevitable. A system of close packed cylinders of uniform radius enclose interspaces amounting to 9 or 22 p.c. of the total bulk of the system according as the axes of the cylinders are arranged in triangles or squares. It is not likely that the muscle fibres are literally close packed, and moreover the blood vessels and possibly also the connective tissue must be added to the interspaces, but on the other hand, any deformation or variation in size of the fibres would lead to a smaller proportion of interspaces. It seems impossible therefore, on the evidence at present available, to decide whether the fraction of the muscle water concerned in the phosphate diffusion corresponds only to the intercellular spaces, or includes certain parts of each cell.

[1] A corroboration of this finding is found in Semeanoff's work on sartorii. Her published data are sufficiently detailed to permit a calculation of the value of α. The values vary from 26 to 56 p.c., with an average value of 45 p.c. This value, though higher than that found in the present work, shows clearly that the whole of the muscle water is not concerned in the exchange of phosphate.

This failure to confirm Stella can be explained satisfactorily, but it is convenient to defer the explanation to a later stage. Meanwhile this rather unexpected result was confirmed by another type of experiment, in which the value of α was determined without previous assumption of the value of c. Pairs of muscles were again used, but in this case one was placed into an approximately equal amount of Ringer's fluid containing a high concentration of phosphate, and the other into the same volume of fluid of low phosphate concentration. Determination of the changes in phosphate concentration in the two Ringer's solutions (and of any slight change in volume due to water exchange between muscle and saline) yielded values for the amount of phosphate diffused into the first muscle and away from the second muscle. If sufficient time was allowed for equilibrium to be attained between the Ringer's fluid and the muscle, the measurements made enabled one to calculate the initial phosphate concentration in the "interspaces" of the muscle as well as the value of α. The only assumptions made in such a calculation were: (1) that when no further exchange of phosphate occurs between muscle and Ringer's fluid, the concentration in any part of the former into which phosphate has been able to diffuse is identical with that of the latter; and (2) that water forms 80 p.c. of the muscle weight. It then follows that

$$\frac{\text{Phosphate initially present in "interspaces"} + \text{Amount of phosphate diffused in from Ringer's fluid}}{\text{Amount of water in "interspaces"}}$$

= Phosphate concentration in Ringer's fluid when equilibrium has been attained.

If α = that fraction of the muscle water contained in the "interspaces,"

c = initial concentration of phosphate in the "interspaces" (in mg. per 100 g. water),

w = weight of muscle initially (in g.),

P = final concentration of phosphate in Ringer's fluid (in mg. per g. water),

x = amount of phosphate diffused into muscle from Ringer's fluid (in mg.),

then
$$\frac{4/5 \times w \times \alpha \times c + x}{4/5 \times w \times \alpha} = P. \qquad \text{.....(1)}$$

When this equation is applied to each muscle of a pair (one in Ringer's fluid of a high phosphate content, one in low) the two unknowns α and c can readily be calculated. In the experiments with living muscles, the "high-phosphate" Ringer's fluid contained 30–60 mg. P per 100 g. and

the "low-phosphate" 5–15 mg. per 100 g.; with muscles in rigor, the values were 150–280 and 0–45 respectively. Phosphate was added in the form of an isotonic solution in order not to affect the total osmotic pressure.

The results of several experiments on resting muscles are given in Table III. A considerable degree of error is to be expected, since the calculations are based on small differences, and the number of measurements made in any one experiment is large (twenty).

TABLE III. The proportion of muscle water occupied by diffusible inorganic phosphate in resting skeletal muscle of the frog.

Group	Duration of preliminary soaking hours	Duration of exp. hours	Proportion of muscle water occupied (α) p.c.
A 1	0	1–1½	28, 22, 32
A 2	0	3	28, 12, 22
B	½	2	25, 15
C	2	21	12, 15, 52
D	1	23	21, 20, 15, 26, 26
			Mean 23 ± 3*
E	44	6	42, 28, 26, 36, 24
F	95	24	30
G	120	19	24
H	1½	65½	33, 32, 27, 28, 42
			Mean 31 ± 2*

For derivation of α see text.

* Probable error of mean.

The proportion of the muscle water occupied by diffusible phosphate in the normal muscle appears to lie between 20 and 30 p.c., and exchange between muscle and Ringer's fluid is restricted to this part of the muscle whether the experiment occupies 1 (in the case of thin muscles such as the sartorius) or 24 hours. The prolonged preliminary immersion in phosphate-free Ringer's fluid of the muscles in groups E–G was undertaken for another purpose, but the equilibrium experiments subsequently performed on them indicate that such prolonged soaking has little effect on the value of α. There is some evidence of a slight increase, but the results are not sufficiently consistent to warrant a definite conclusion on the matter. The only values of c which can be compared with those given in Table I are from group A; these were 17, 12, 17, 12, 9 and 13 mg. P per 100 g. water in α respectively. In the other groups, in which the muscles were immersed in phosphate-free Ringer's fluid for varying periods of time before the equilibrium experiment was performed, the value of c was naturally lower (practically zero in groups E–G).

This type of experiment therefore confirms those given earlier in leading to the conclusion that phosphate from a surrounding Ringer's fluid will diffuse freely into only 20–30 p.c. of the muscle water in the case of resting muscles, and that the phosphate concentration in this fraction is of the order of 15 mg. per 100 g. water.

Muscle-bone preparation.

It is convenient at this point to consider why Stella found, with thigh preparations, the value of the fraction α to be between 50 and 80 p.c., whilst preparations of isolated muscles give values in the neighbourhood of 25 p.c. Experiments were performed with thigh preparations of different sizes, the technique being similar to Stella's. His thigh preparations weighed about 3 g. each. The smallest preparation used in the present work weighed 5 g. It gave a value of α of 60 p.c. (Table IV). Larger

Table IV. Diffusion of inorganic phosphate (I.P.) into the resting thigh preparation of the frog.

Exp.	Duration of exp. hours	Wt. of muscle g.	Wt. of Ringer's fluid g.	I.P. conc. in Ringer's fluid finally mg. per 100 g.	I.P. diffused into prep. mg.	α p.c.
1	24½	25·8	30	74·4	3·0	24
2	24	16·3	18·5	61·0	1·99	31
3	19	12·7	17·0	81·8	2·07	30
4	18	10·4	18·5	62·5	1·55	37
5	18	7·4	17·0	41·6	0·73	41
6	23½	6·5	17·2	76·3	1·7	51
7	24	5·5	18·0	84·0	1·55	53
8	24	5·0	20·0	47·6	0·828	60

The fraction of the muscle water occupied by diffusible phosphate (α) is calculated on the basis of an assumed initial concentration in this fraction of 12½ mg. per 100 g. water (Table I). For details see text. In Exps. 1–6 Hungarian frogs (*R. esc.*) were used, and in 7 and 8 Dutch frogs (*R. esc.*).

preparations yielded lower values of α. Indeed it became obvious that the apparent value of α varied inversely with the size of the preparation, and suggested at first sight that insufficient time had been allowed for phosphate equilibrium to be established between these larger preparations and the surrounding saline. The actual rate of diffusion of phosphate into preparations of different sizes was therefore determined. Two such cases are depicted in Fig. 1, curve I showing the rate of diffusion into a preparation containing 25 g. of muscle, and curve II into one containing 5 g. of muscle. In both a steady rate of phosphate entrance is reached between 12 and 18 hours, the magnitude of which (per g. of tissue) varies inversely with the size of the preparation. Further light is thrown on the matter by investigation of the behaviour of a bone preparation (a

thigh preparation with all the muscle removed) exposed to such Ringer's fluids. It is seen (curve III, Fig. 1) that phosphate diffuses into such a preparation continuously over the periods in question. The results quoted in Table V show equally clearly that bone is capable of absorbing large

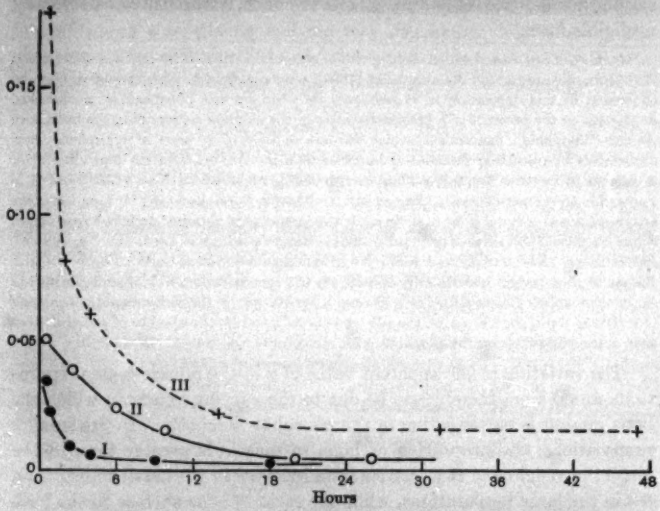

Fig. 1. Rate of diffusion of inorganic phosphate into muscle-bone and bone preparations. Curve I, muscle-bone preparation containing 25 g. muscle. Curve II, muscle-bone preparation containing 5 g. muscle. Curve III, bone preparation. The concentrations of phosphate in the Ringer's fluid surrounding the preparation at the end of the experiment were 74, 84 and 68 mg. P per 100 g. respectively. Ordinates: rate of diffusion in mg. P per g. tissue per hour.

TABLE V. Diffusion of inorganic phosphate (I.P.) into and out of bone.

Exp.	Duration of exp. hours	Wt. of bone g.	I.P. conc. in Ringer's fluid finally mg. P per 100 g.	Change in I.P. content of bone mg.
A	24	3·0	41·9	+0·585
B	23	2·9	41·4	+0·94
C	47	2·3	67·7	+3·15
D	23	2·6	1·6	−0·18
E	20	1·0	4·5	−0·42

In Exps. A–D the thigh preparation freed from muscle was used (two preparations in Exp. C); in Exp. E intact femurs (4). Hungarian frogs (*R. esc.*) were used in Exps. A, B and D; Dutch frogs (*R. esc.*) in Exps. C and E.

amounts of phosphate from surrounding Ringer's solutions. Attempts to express the behaviour of the Ringer-bone system in terms of simple diffusion lead to impossibly high values for the water content of the bone —200 p.c. by weight in one experiment. This is a strong indication that the phosphate, once it has diffused into the bone, is stored in an osmotically negligible form.

Much work has been done on the deposition of insoluble phosphate in slices of rat bone. Robison, Macleod and Rosenheim [1930] conclude that two mechanisms may be concerned in this deposition in experiments *in vitro*: (*a*) the phosphatase mechanism, dependent on the presence of a phosphoric ester in the medium surrounding the bone, and (*b*) the "inorganic" mechanism, which favours deposition in bone of phosphate from supersaturated solutions: the relative importance of the two mechanisms depending upon a number of factors. The subject has been recently reviewed by Kay [1932], but it is impossible to compare directly the present results with those discussed by him, since the latter concerned only rat bone in slices while the former were obtained on intact frog bones. Since no phosphoric ester was present in the Ringer's solutions used, the "inorganic" mechanism must be entirely responsible for phosphate deposition in these experiments, and the controlling factors undoubtedly include (1) the concentration of inorganic phosphate in the surrounding Ringer's fluid (40–100 mg. P per 100 g.), (2) the concentration of calcium ions (10 mg. $CaCl_2$ per 100 g.), (3) the *p*H (usually 8·0), and (4) the absence of protein, other than traces diffused from the preparation.

The variation in the apparent value of α in the muscle-bone preparations would seem therefore to be due to the varying extent to which the bone present is participating in the phosphate exchange. In the smaller preparations, the proportion of bone to muscle is greater than in the larger; in weight the ratio varies from over 0·3 in the small to less than 0·2 in the large preparations, while the ratio of bone surface to the bulk of muscle will vary in the same direction and to a greater extent. The rate of diffusion of phosphate will also tend to be lessened in the larger preparations by the mass of tissue to be traversed between Ringer's fluid and bone, since the diffusion coefficient of phosphate in muscle is less than one-twentieth of that in Ringer's fluid [Stella, 1928].

It is impossible to assess exactly the true value of α in the muscles of these preparations, but it seems likely that it is not materially different from that found in isolated muscles, since the larger the preparation (that is, the greater the proportion of muscle to bone) the more nearly does the value of α approximate to that of isolated muscle.

Rigor muscles.

In the equilibrium experiments already quoted, there was no significant movement of water between muscle and Ringer's fluid, but when the behaviour of dead muscles was investigated, such movement was

considerable and could not be ignored. The question then arose as to whether all parts of the muscle were sharing equally in this water exchange, or whether any one fraction was playing the major part. If water exchange concerned only that part into which phosphate could not readily diffuse, viz. $(1-\alpha)$, then the equation already used for the calculation of α and c in resting muscles was still valid. But if water and phosphate were diffusing into and from the same part of the muscle, equation (1) would no longer apply; it would have to become

$$\frac{4/5 \times w \times \alpha \times c + x}{4/5 \times w \times \alpha + y} = P, \qquad \text{.....(2)}$$

where y = the amount of water entering the "interspaces" of the muscle.

A series of equilibrium experiments was therefore performed, on both resting and dead muscles, in order to determine which equation yielded the more consistent results; movement of water was magnified in the experiments on resting muscles by the use of hypo- and hypertonic Ringer's fluids. The results given in Table VI show that in the case of living, but not of dead, muscles the use of equation (1) is fully justified.

TABLE VI. Diffusion of inorganic phosphate into frog sketal muscle from hyper- and hypotonic Ringer's fluid.

Exp.	State of muscle	Ringer's fluid	α_1 p.c.	α_2 p.c.
A	Resting	1·75 normal	29	49
B	,,	,,	30	48
C	,,	0·5 normal	29	6
D	,,	,,	18	−1
E	Rigor. Frog kept 6 hours at 42½° C.	3·0 normal	99	95
F	,,	,,	110	96
G	,,	,,	131	104
H	,,	,,	119	105
I	,,	,,	132	107

α is the proportion of the muscle water occupied by diffusible phosphate. α_1 is calculated on the hypothesis that water exchange concerns only the remaining part $(1-\alpha)$ of the muscle water; in the calculation of α_2 it is assumed that this fraction itself (or a part of it) is concerned in the exchange of water. For the method of calculating see equations (1) and (2).

In regard to these results on dead muscles, two points of interest emerge: (*a*) phosphate can now diffuse into the whole of the water of the muscle; and (*b*) into whatever part of the muscle water is drawn by osmotic forces, phosphate can also diffuse[1]. The application of equation (1) to such muscles yields an impossibly high value for α.

These results were corroborated by others of a more direct type. Either the muscles (dissected after the frog had passed into rigor) were

[1] The part of the muscle which swells or shrinks is probably still the "cells," that is the fraction $1-\alpha$ of living muscles, but this is now included in that part of the muscle containing diffusible phosphate.

soaked several hours in a phosphate-free Ringer's fluid—analysis of some of them at this stage showing a phosphate content equal to that in the fluid outside—and then transferred to a Ringer's fluid of high phosphate content; or they were placed without preliminary soaking into a Ringer's fluid containing either more or less phosphate than themselves. In all cases the phosphate content of the muscle and of the Ringer's fluid was determined at the end of the experiment, and also the degree of water exchange between the two. The results of several such experiments are given in Table VII. In most cases the muscles gained water during the experiment, but in two (C 1 and C 2) movement in the opposite direction was induced by addition of cane sugar to the Ringer's fluid. The results of this set of experiments (C) corroborate those given by the less direct method (Table VI). In Exps. A and B, however, the whole water of the muscle does not seem to be available for dissolving phosphate diffusing in from the surrounding saline. But if the water initially present in the muscle is alone assumed to be available, then impossibly high values are obtained for α (110–120 p.c.). It would appear that in these muscles the breakdown of barriers to phosphate diffusion is nearly, though not yet quite, complete.

TABLE VII. Diffusion of inorganic phosphate (I.P.) into the skeletal muscles of the frog in rigor (measured by direct analysis of muscles).

Exp.	Type of exp.	Duration of exp. hours	Initial water content of muscle g.	Water gain by muscle g.	I.P. content of muscle finally mg.	I.P. conc. in muscle water finally mg. per 100 g.	I.P. conc. in Ringer's fluid finally mg. per 100 g.	α p.c.
A 1	Initial content of	1	0·101	0·031	0·1253	95	128	74
2	I.P. in muscle re-	2	0·132	0·02	0·1723	113½	125	91
3	duced to 5 mg.	2	0·133	0·045	0·187	105	125	84
4	per 100 g. water	4	0·183	0·067	0·276	110½	125	89
B 1	Initial content of	18½	0·57	0·132	0·763	109	120	91
2	I.P. reduced to	18½	0·572	0·112	0·76	111½	120	93
3	about 10 mg. per	18½	0·91	0·271	1·302	110½	120	92
4	100 g. water	18½	0·717	0·146	0·924	107	120	89
C 1	Initial content of	17	0·576	−0·023	0·709	128	127½	100
2	I.P. about 85 mg.	17	0·581	−0·014	0·3745	66	62½	105
3	per 100 g. water	17	0·469	0·012	0·642	133½	130	102
4		17	0·52	0·009	0·342	64½	62½	103

α is the ratio of the two preceding columns.

In Exps. C 1 and 2 hypertonic Ringer's fluid was obtained by addition of 15 p.c. cane sugar. This appreciably affected the specific gravity of the fluid, for which due allowance was made in calculating the phosphate concentration per 100 g. of water.

In fully advanced rigor therefore, the evidence from both direct and indirect types of experiment indicates that the whole of the muscle water,

including the water which passes into the muscle from the surrounding saline during the course of the experiment, is available to dissolve phosphate. This does not necessarily entail a mechanical rupture of all the membranes in the muscle, since the dead muscle swells in salt solution and the swelling can be prevented by cane-sugar solution. These may be still membranes relatively impermeable to cane sugar and to some constituents, including probably the protein of the muscle "cells," but it may be that the ability of cane sugar to prevent swelling is due to its slow diffusion, relative to water.

Muscles other than in the resting state or in heat rigor.

Values for α and c were also determined in muscles other than those resting or in rigor. The results (Table VIII) were calculated by the use of equation (1); little movement of water between muscle and Ringer's fluid occurred in any of them, however, so that the results would not be appreciably altered had equation (2) been applied. No changes comparable with those observed in rigor are to be observed in the value of α.

Table VIII. The effect of various treatments upon the concentration of diffusible inorganic phosphate (I.P.) in frog skeletal muscle and the proportion of muscle water occupied by it.

Group	Condition of muscles	Duration of exp. hours	Proportion of muscle water occupied (α) p.c.	Conc. (c) of I.P. in α mg. per 100 g. water
A 1	Fatigued *in situ* before	3–5	15, 11, 20, 46, 24	20, 18, 24, 17, 20
A 2	dissection	3	22, 26, 31	19, 24, 25
B	" Greater fatigue	2½–5	12, 17, 33, 44, 19	21, 31, 35, 27, 32
C	Anaerobiosis	17	26, 24, 33, 28	21, 14, 38, 24
D	H_2 + coal gas 23 hours at 16° C.	2½–5	36, 32, 28, 49	47, 23, 28, 27
E	H_2 + coal gas during exp.	17	11, 30, 34, 41, 59	66, 45, 42, 48, 40
F	Resting: initial immersion in Ringer's fluid containing 45½ mg. I.P. per 100 g.	16	21, 30	45, 48

In groups A, B and C the hydrogen used was prepared from zinc: in groups D and E the hydrogen used (from a cylinder) smelt strongly of coal gas. In all experiments the value of α was obtained by use of equation (1).

There is a slight tendency for the "interspaces" to be restricted in fatigue (Exps. A and B), and this would entail a relative increase in the remaining part of the muscle. One would expect such a result if the chemical breakdown products of fatigue were unable to diffuse out freely

and rapidly from the muscle "cells," but the present results are not sufficiently consistent to warrant a definite conclusion to this effect. Anaerobiosis in its early stages (17 hours at 2–3° C.) had no appreciable effect (Exp. C). In Exps. D and E hydrogen was used which was subsequently found to be contaminated with coal gas; the muscles were found to be inexcitable at the end of the experiment, and the value of α had increased.

The interest of the experiments in group F lies particularly in the value of c. The pairs of muscles used had been kept for 23 hours in an oxygenated Ringer's fluid containing 45½ mg. P per 100 g. They were then wiped and reweighed, and the value of c determined for each pair by means of an equilibrium experiment. The values obtained (45 and 48 mg. P per 100 g. water respectively) show clearly that the "interspaces" (containing 21 and 30 p.c. of the muscle water respectively) had come completely into equilibrium with the surrounding Ringer's fluid, and add to one's confidence in the physical meaning of the value of c obtained in other experiments.

The value of c is also of special interest in the experiments (groups A and B) on fatigued muscles. The muscles were stimulated *in situ*, series of tetani being produced by use of an induction coil, the treatment being continued for several minutes. In group B greater fatigue was induced than in group A. Direct analysis of the inorganic phosphate content of these muscles (group B) at the end of the equilibrium experiment yielded a value of 51 mg. P per 100 g. muscle water, which, taken in conjunction with the equilibrium value of 29 mg. per 100 g. in 25 p.c. of the muscle water, indicates a value of 58 mg. per 100 g. in the remaining 75 p.c. of the water. Due care was taken to avoid stimulation of the muscles afresh while they were being killed (by the use of ice-cold trichloracetic acid), and the protein-free filtrate was immediately neutralized with baryta to prevent breakdown of phosphagen. It is unlikely therefore that this value of 51 mg. P per 100 g. muscle water is fictitiously high.

A similar, though smaller, difference between the values found for the phosphate content of skeletal muscle by direct analysis and by equilibrium experiment is found in the case of resting muscles. The lowest figures yielded by direct analysis vary from 12½ to 25 mg. per 100 g. muscle water [Eggleton and Eggleton, 1929], whereas equilibrium values vary from 10 to 15 mg. per 100 g. water. It seems probable therefore that one-quarter of the muscle water contains phosphate which can diffuse away from the muscle into Ringer's solution, and that the remaining three-quarters of the muscle water contains phosphate in about twice the

concentration, restrained by semipermeable membranes from diffusing out so long as the muscle is alive, and at rest.

The value of 51 mg. per 100 g. water (only 41 mg. per 100 g. when expressed as a proportion of the muscle and not of its water content) is, however, lower than those previously found by direct analysis of the muscle. In the present series of experiments ample time for anaerobic resynthesis of phosphagen was given, whereas in the earlier recorded work [Eggleton and Eggleton, 1927] the muscles were killed immediately after stimulation had ceased, and this difference in treatment is the most likely cause of the difference in results.

Exchange of phosphate between "cells" and "interspaces" in the muscle.

The foregoing experiments lead to the conclusion that in respect of the exchange of phosphate between itself and Ringer's fluid the behaviour of a living muscle is identical with that of a quantity of water equal to a fraction (α) of the total water of the muscle, containing phosphate in a concentration c. In so far as description in these terms is successful there can be no exchange of phosphate between the two fractions of the water of the muscle: the fact that the numerical value of α is found to be the same within the limits of experimental error for experiments of 2 hours' duration as for those of 24 hours implies that any exchange of phosphate between "cells" and "interspaces" must be subject to far greater hindrance than exchange between the "interspaces" and the Ringer's fluid outside. Yet in the experiments on fatigued muscles and muscles maintained under anaerobic conditions, the value of c is found to be higher than that in resting muscles. There are several possible explanations of this result.

(1) An increase in c would result from a decrease in the size of the "interspaces" due to withdrawal of water into the remainder of the muscle by osmotic forces. A minute decrease in α is sometimes observed as a result of fatigue, but the decrease of 50 p.c. which would be required on this hypothesis to account for the doubled value of c is never found.

(2) An increase in c might result from the breakdown of some phosphoric ester present in the "interspaces." But it is generally agreed that all inorganic phosphate formed within physiological limits in muscle is derived from phosphagen. (The hexosephosphate and the adenylpyrophosphate, which account for practically all the organic phosphate other than phosphagen, give rise to no inorganic phosphate within physiological

limits of fatigue or oxygen lack.) But the inability of phosphagen to diffuse from muscle seems to indicate that it is not contained in the same fraction as the diffusible inorganic phosphate (*i.e.* the "interspaces").

(3) It seems more likely that in certain circumstances and within certain limits phosphate can diffuse from the "cells" to the "interspaces." Such a diffusion was observed in isolated oxygenated muscles when the surrounding Ringer's fluid was maintained practically phosphate-free by frequent renewal, but the rate of diffusion was so minute as to be inappreciable within 24 hours, and throws no obvious light on the diffusion observed in muscles fatigued or suffering from oxygen lack. It may be, as Embden suggested in 1921, that membranes become more permeable when the muscle is in the active state; it is also conceivable that the membranes concerned maintain, whenever possible, a constant ratio of phosphate on the two sides, and that diffusion outwards into the "interspaces" occurs to some extent whenever the concentration of phosphate inside the "cells" is increased or that in the "interspaces" lowered. Whatever be the underlying mechanism of the action of such semipermeable membranes, the evidence available indicates that this property of the membranes serves greatly to hinder diffusion outwards of inorganic phosphate produced within the muscle "cell."

To this property of the muscle "cell" boundary may perhaps be ascribed the observation that the increase in concentration of inorganic phosphate in the blood of an exercising animal is relatively small in comparison with the increase in the muscles themselves. Havard and Reay [1926] found only a small rise in the concentration of inorganic phosphate in the blood of athletes, excercised to the point of exhaustion; we have no direct knowledge of the concentration of this ion in the muscles themselves under such conditions, but in view of the large increase in the concentration of lactate in the blood it seems probable that the muscles were inadequately oxygenated. Direct determination of the phosphate concentration in blood and muscle of artificially exercised anæsthetized cats confirms this interpretation. The technique employed in such experiments has been described elsewhere [Eggleton and Evans, 1930]. These workers found (unpublished experiments) that stimulation sufficiently severe to cause an increase of 140 mg. lactic acid per 100 g. blood, led to an increase in its inorganic phosphate concentration of only 6 mg. P per 100 g., although the muscles analysed at the same time showed increases of 40–80 mg. P per 100 g. tissue. In lesser degrees of fatigue the same relationship between the increase in concentration of phosphate in muscle and blood was still observed. In such experiments

the major part of the musculature of the body was caused to contract, so that the volume of circulating blood was considerably less than that of active muscle, and equilibrium between the two should have been rapidly established. It might be argued that in such intact animals, phosphate was diffusing out from the muscles into the blood stream and being as rapidly removed by other tissues such as the kidney and bone. But no perceptible decrease in the total acid-soluble phosphate content of such muscles was apparent after fatigue, and one is driven to the conclusion that the muscle cell membranes in the body permit very little inorganic phosphate to pass through, despite a high concentration of this ion within the cell.

Summary.

1. When an excised frog muscle is immersed in Ringer's fluid until equilibrium is reached in respect of phosphate exchange (2–5 hours at 2–3° C.) analysis of either muscle or Ringer's fluid indicates that only 20–30 p.c. of the water of the muscle has been involved in the diffusion system. It is therefore suggested that the muscle cells are bounded by membranes which are impermeable in these circumstances to phosphate.

2. The different result previously obtained on the thigh preparation by Stella is due to the fact that the bone present can take up or give out considerable quantities of inorganic phosphate, thereby masking the capacity of the muscle tissue itself.

3. In heat rigor, the whole of the water of the muscle becomes ultimately freely available to phosphate diffusing in from a surrounding Ringer's fluid; the phosphate contained initially in such a muscle will also diffuse out freely and rapidly.

4. In other conditions of the muscle (fatigue, etc.) little change is observed in the relative size of the "interspaces." When the muscle is poisoned with coal gas a definite increase is noted.

5. Under the conditions in which these experiments were performed the phosphate concentration with which resting muscles (isolated muscles or thigh preparations) are in equilibrium lies between 10 and 15 mg. P per 100 g., while the phosphate concentration with which fatigued muscles are in equilibrium is about 30 mg. P per 100 g. Such fatigued muscles on direct analysis yield a value of 50 mg. P per 100 g. muscle water. These results are discussed in relation to those obtained by Stella and Semeanoff.

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CAROTID SINUS REFLEXES. INFLUENCE OF CENTRAL BLOOD-PRESSURE AND BLOOD SUPPLY ON RESPIRATORY AND VASO-MOTOR CENTRES[1].

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The reflex and tonic influences of the pulmonary vagus on the activity of the respiratory centre is a well-known and generally accepted fact [Hering and Breuer, 1868; Head, 1889; Haldane, 1922; J. F. and C. Heymans, 1926; C. Heymans, 1928; Hoffmann and Keller, 1929; Hess, 1931; Anrep and Adli Samaan, 1932]. It has been shown by J. F. and C. Heymans [1926] and C. Heymans [1928, 1929*a*, *b*] that the vago-depressor nerves in dogs are also the centripetal paths of respiratory reflexes in relation with the cardio-aortic blood-pressure; an increase of the arterial pressure in the region of the left ventricle and the aortic arch producing a reflex inhibition of the respiratory centre, while a lowering of blood-pressure in those districts provokes a reflex hyperpnœa. The normal arterial pressure in the left heart and aortic arch maintains a reflex tonus on respiration. The experiments of Moissejeff [1927], C. Heymans [1928, 1929*a*, *b*], Florey, Marvin and Drury [1928], Bouckaert and C. Heymans [1930], C. Heymans, Bouckaert and Dautrebande [1932], Wright [1930], Koch and Mark [1931], Gollwitzer-Meier and Schulte [1931], and Schmidt [1932] have demonstrated that the pressure in the carotid sinus, the functional homologue of the aortic area, also reflexly regulates the activity of the respiratory centre, by way of the carotid sinus nerves. An increase of blood-pressure in the carotid sinus produces a reflex inhibition of the respiration, or even apnea; a decrease of blood-pressure in the same vascular area produces a reflex hyperpnœa.

[1] Preliminary reports: *C. R. Soc. Biol.*, Paris, 1932, 110, 996; 111, 145. *Sunti delle Comm. Scientif. Congr. intern. Fisiologia*, 1932, p. 117.

The rôle and the importance of the cerebral blood flow in the regulation of respiration and circulation have been emphasized by numerous workers [François-Franck, 1877; Marey, 1881; Hédon, 1910; Chabrol, 1922; Gesell, 1923; Roberts, 1924; Anrep and Starling, 1925; C. Heymans and Ladon, 1925; Gesell, 1929; C. F. Schmidt, 1928, 1929, 1932; C. Heymans, 1928; C. Heymans and Bouckaert, 1930; Wright, 1930; C. Heymans, Bouckaert and Dautrebande, 1931, 1932].

It is known that, in normal animals, occlusion of the common carotid arteries stimulates, while the reopening of the same arteries inhibits, the activity of the respiratory centre [Cooper, 1836; Kussmaul and Tenner, 1855; Hill, 1896; Lumsden, 1923; J. F. and C. Heymans, 1926; Gesell, 1929; Schmidt, 1929].

These facts were taken, especially by Gesell and Schmidt, as evidence that the respiratory centre is influenced and regulated directly by changes in the central blood-pressure and blood supply. The same theory of regulation by central blood-pressure and blood supply has been defended [François-Franck, 1877; Hédon, 1910; Chabrol, 1922; Anrep and Segall, 1926; Nash, 1926] for the regulation of the activity of the cardio-regulatory and vaso-motor centres.

Pagano [1900], however, showed that although the occlusion of the common carotids provokes a stimulation of the vaso-motor and cardio-accelerator centres, the same results do not follow after occlusion of the vertebral arteries and the efferent branches of the common carotids; he concluded that the regulation of the vaso-motor and cardio-regulatory centres is effected reflexly, rather than by a direct action on the centres.

Roberts [1924] concluded from his experiments that the respiratory centre is totally insensitive to diminution in its blood supply, until this diminution becomes extreme.

Numerous investigations have shown, by various experimental methods, that the changes in the activity of the respiratory centre [C. Heymans and Bouckaert, 1930; Koch and Mark 1931; C. Heymans, Bouckaert and Dautrebande, 1932], as well as the changes in the tonus and activity of the heart regulatory and vaso-motor centres [Hering, 1927; C. Heymans, 1928; Florey, Marvin and Drury, 1928; C. Heymans, 1929 *a*; C. Heymans and Bouckaert, 1930; Wright, 1930; Koch, 1931; C. Heymans, Bouckaert and Dautrebande, 1931, 1932; C. Heymans, Bouckaert and Regniers, 1933] produced by the modifications of blood-pressure and blood flow in the cephalic arterial circulation are not due to changes in the central

blood-pressure, but to the blood-pressure reflexes arising in the carotid sinus. These experiments have shown, indeed, that in dogs the occlusion of neither the centrifugal branches of both carotid sinuses, nor the occlusion of the two vertebral arteries elicited any changes in the activity of the respiratory or cardio-regulating or vaso-motor centres. On the other hand, it has been shown that slight changes of blood-pressure in the carotid sinus produce definite reflex changes in the activity of the respiratory and circulatory centres.

In spite of these clear experimental proofs, obtained by several experimental methods on animals in good general circulatory and respiratory conditions, the theories of direct regulation of respiratory and circulatory centres by changes of blood-pressure and blood supply are still maintained and defended by several workers. Thus, Anrep and Segall [1926], Nash [1926] and Volhard [1930] concluded that the cephalic control of the heart rate by blood-pressure changes does not depend exclusively on the carotid sinus reflexes, but also upon another more central effect. Raab [1932] also says that a slight decrease of the cerebral blood supply directly stimulates the vaso-motor centre. Recently, Schmidt [1932] has reinvestigated the problem of the effect of changes of cephalic blood-pressure and blood flow on the activity of the respiratory centre, and although he generally confirms our observations and conclusions concerning the effects of carotid sinus reflexes on the respiratory centre, he also concludes from his experiments that the respiratory centre is directly affected by alterations in its blood supply, and that the respiratory effects of alterations in cephalic blood-pressure may be due either to carotid sinus reflexes or to alterations in central blood flow, or to both. Schmidt believes that influence of change in the central blood flow is quite as sensitive as, and decidedly more powerful than the reflex influence, but he makes the reservation that the sensitivity of the central mechanism is more variable than the reflex one.

Considering the importance of the problem for the physiology of respiration and circulation, and in order to submit our previous conclusions to a new and still more severe control, we have performed a new series of experiments with new methods, now to be described.

Experiments.

Method (Fig. 1). Dogs are anæsthetized with choralosane (orthochloralose, 0·8 g. pro kg. intravenous); this anæsthetic maintains a good respiration and a normal circulation. One carotid sinus is prepared following the technique of Moissejeff [1927]: the large efferent (centri-

fugal) branches of the carotid sinus are ligaturated, its innervation remains intact; thus the blood of this common carotid artery may perfuse the carotid sinus through the more minute efferent branches, and so the carotid sinus pressure may be changed by compression and decompression of the carotid artery which may be done without directly changing the central blood-pressure and blood flow. The carotid sinus of the other side is denervated, and after ligature of all the efferent branches of the carotid

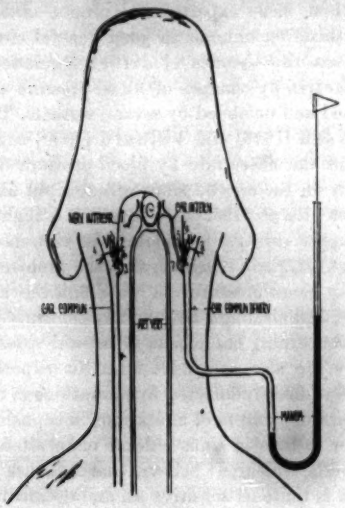

Fig. 1.

sinus except the internal carotid, the central end of the common carotid artery is connected with a mercury manometer, in order to measure the pressure in the circle of Willis, *i.e.* the intra-central arterial blood-pressure, following the technique of Hürthle [1889]. The two vertebral arteries are isolated, the general arterial blood-pressure is registered in the femoral artery, and the respiratory plethysmogram is registered. With this method it is possible to modify the internal carotid sinus pressure, or to clamp the vertebral arteries, and at the same time to record the changes in central blood-pressure, in general arterial blood-pressure and in the respiratory movements.

Results.

(1) *Effects of occlusion and release of the vertebral arteries upon the central blood-pressure, and upon the activity of the respiratory and vaso-motor centres.*

The curves of Fig. 2 A show that in a dog prepared by the method described above, the occlusion of both vertebral arteries provokes an immediate and abrupt fall of the central blood-pressure from 80 to 40 mm. Hg; but the general blood-pressure, and the activity of the vaso-motor and the respiratory centres are unaffected, although the central blood-pressure remains low throughout the vertebral occlusion, during which time the central circulation is only provided by the spinal arteries.

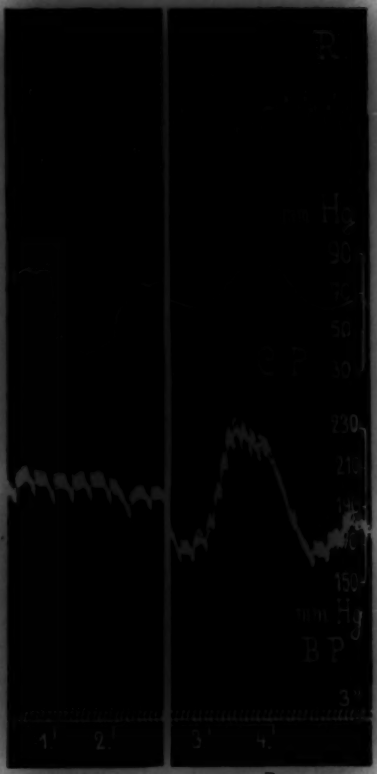

Fig. 2. Dog prepared following the method of Fig. 1. Upper record, pneumogram (*R*). Middle record, central blood-pressure (*C.P.*). Lower record, general arterial blood-pressure (*B.P.*). At 1, occlusion of the two vertebral arteries. At 2, re-opening of the two vertebral arteries. At 3, lowered pressure in the circulatory isolated carotid sinus. At 4, normal pressure restored inside the isolated carotid sinus.

These experiments demonstrate that the occlusion of the vertebral arteries does not cause general hypertension (in agreement with Pagano [1900], C. Heymans [1928], Florey, Marvin and Drury [1928]) or hyperpnœa, although the central blood-pressure is very much reduced; further, that the release of the same arteries does not influence the vaso-motor or respiratory centres, although the central blood-pressure is now much increased.

Schmidt [1932] has recently confirmed this observation; he has also measured the average decrease of central blood flow, and found a reduction of 25 p.c. in the cerebral flow on clamping the vertebral arteries in dogs. Schmidt believes that the absence of hyperpnœa under these conditions may merely mean that the anæmia so produced was not great enough. We believe, if a reduction of cerebral blood flow must be greater than 25 p.c. before the respiratory centre responds to it, that such a mechanism has no proper physiological signification or importance as a means of central regulation of the activity of the respiratory centre. The same restriction applies to the direct sensitivity of the vaso-motor centre to changes of central blood-pressure and blood flow.

In a second group of experiments we have re-investigated the influences of carotid sinus pressure upon the central blood-pressure and blood flow and upon the activity of the medullary centres.

(2) *Effects of carotid sinus pressure upon the central blood-pressure, cerebral blood flow, and activity of the respiratory and vaso-motor centres.*

(*a*) *Carotid sinus pressure and central blood-pressure.*

The same method (Fig. 1) was used, and a typical result is shown in Fig. 2 B. By clamping the common carotid, the pressure in the isolated but innervated carotid sinus is lowered; the blood inside the carotid sinus indeed escapes through the small arterial branches which were not ligatured, but the occlusion of this common carotid has no direct influence on the central blood supply. The curves show that the decrease of pressure in the isolated carotid sinus produces an immediate reflex general arterial hypertension and a marked hyperpnœa, although the central blood-pressure is at the same time very much increased from 65 to 88 mm. Hg. The increase of carotid sinus pressure at 4, Fig. 2 B, produces an immediate reflex fall of general blood-pressure with a marked decrease of central blood-pressure and an inhibition of the respiratory centre.

The results of another typical experiment are shown in the curves of Fig. 3. Here, lowering of the central pressure by clamping the two vertebral arteries (1–2, Fig. 3) has no effect on general circulation or respiration, whereas lowering of the carotid sinus pressure (3–4, Fig. 3) provokes a reflex general hypertension and a stimulation of the respiratory centre, in spite of an attendant increase in the central blood-pressure from 55 to 75 mm. Hg. This reflex stimulation of vaso-motor and respiratory centres persists during the 7 min. of low carotid sinus pressure and high central pressure; the return to normal carotid sinus pressure at 4, Fig. 3, provokes

an immediate reflex inhibition of the vaso-tonic and respiratory centres, together with a fall of the central pressure. These experiments clearly demonstrate that a considerable reduction of the central blood-pressure by clamping the vertebral arteries has no direct stimulating effect on the respiratory and vaso-motor centres, while on the contrary a decrease of the carotid sinus pressure produces reflex stimulation of the vaso-motor and respiratory centres, although the central blood-pressure is thereby increased.

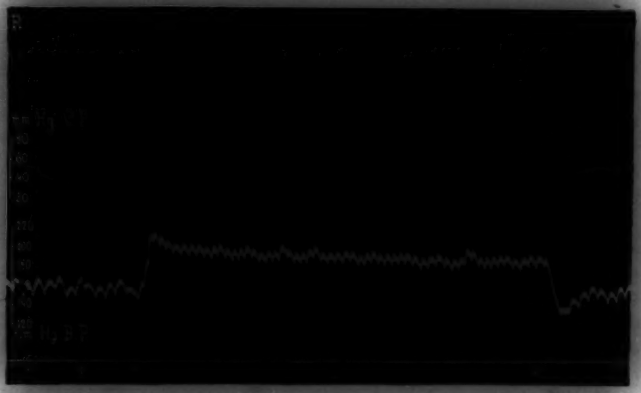

Fig. 3. Dog prepared following the method of Fig. 1. Upper record, pneumogram (*R*). Middle record, central arterial blood-pressure (*C.P.*). Lower record, general arterial blood-pressure (*B.P.*). At 1, occlusion of the two vertebral arteries. At 2, reopening of the two vertebral arteries. At 3, low pressure in the isolated but innervated carotid sinus. At 4, normal pressure in the carotid sinus.

(*b*) *Carotid sinus pressure, central blood flow and cerebral vaso-motor tonus.*

The influence of the carotid sinus reflexes upon the vascular tonus in the centres themselves, and upon the cerebral blood flow, has been further investigated.

The problem of the regulation of the cerebral blood flow, and the question whether the cerebral blood vessels possess an active vaso-motor innervation, or whether they, and hence the cerebral blood flow, are, on the contrary, passive and dependent only upon the general blood-pressure, have long been the subjects for discussion and investigation. Nothnagel [1867], Schuller [1874], Vulpian [1875], Cavazzani [1893], Wie-

chowski [1902, 1905], Muller and Siebeck [1907], Kurusu [1928], Forbes [1928], Forbes and Wolff [1928], conclude in favour of the existence of cerebral vaso-motor nerves; on the contrary, Schulz [1866], Riegel and Jolly [1871], De Boeck and Verhoogen [1890], Roy and Sherrington [1890], Bayliss, Hill and Gulland [1895], Hill and Macleod [1900], Florey [1925] and others do not accept the existence of an autonomic vaso-motor cerebral regulation. According to Cobb [1929] it has been shown that, physiologically, pial vessels may constrict or dilate in response to appropriate stimulation, but Hill's statement, strictly speaking, still holds: "There is no evidence of a causation of cerebral anæmia by spasm of the cerebral arteries."

Our previous experiments [1928] have shown that the blood vessels arising from the external carotid artery, the cephalic peripheral arteries, react to the carotid sinus vaso-motor reflexes in the same way as the muscular and splanchnic arteries. The same fact has been shown by Anrep and Segall [1926], and ourselves [1928], for the aortic-depressor reflexes.

Rein (1929, 1931) has shown that clamping of one common carotid artery produces a definite increase of blood flow through the other common carotid. He attributes this change of cephalic blood flow to a reflex vaso-dilatation in the cephalic, cerebral, circulation, caused by the low carotid sinus pressure. But several factors of mechanical and circulatory origin could be involved in this change of one carotid blood flow after cutting off the blood flow in the other common carotid.

Keller [1930] concludes from his experiments that the blood flow through the internal carotid is in general "druckpassiv" in dogs, but he also accepts an autonomic peripheral regulation of the cerebral vessels and the cerebral blood flow.

It was found by Gollwitzer-Meier and Schulte [1932] that on raising the arterial pressure in the isolated carotid sinus, the reflex fall of general arterial pressure is associated with an initial constriction and a secondary dilatation of the arteries of the retina. The constriction is accepted to be a passive effect and the dilatation an active reflex vaso-motor reaction. It is thought that the vessels of the brain participate actively to the general reflex regulation of the blood-pressure in a similar manner. Several objections may be made to these experiments and conclusions, and we therefore think that the question of the influence of the carotid sinus reflexes on the cerebral vessels and especially on the cerebral blood flow needs to be re-examined.

I. Carotid sinus pressure and central blood flow.

Method (Fig. 4). Dogs were anæsthetized with chloralosane, one carotid sinus was isolated from the circulation, its innervation remaining intact; the pressure may be modified artificially in this isolated but

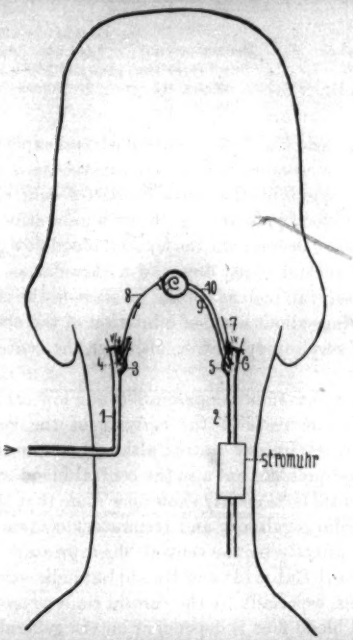

Fig. 4. 1–2, common carotid arteries. 3, ligaturated internal carotid artery with normal carotid sinus innervation. 4, ligaturated external carotid artery. 5, denervated carotid sinus. 6, ligaturated external carotid artery. 7, carotid sinus nerve cut. 8, carotid sinus nerve with normal central connections. 9–10, internal carotid and occipital arteries connecting the denervated carotid sinus and the common carotid blood stream with the central nervous system.

innervated carotid sinus (technique of Moissejeff [1927]. The carotid sinus on the other side was denervated and the external carotid artery ligatured, the blood flow in this common carotid artery (internal carotid

and occipital artery supplying the cerebral circulation), and in a femoral artery, was measured by means of a mechanical Stromuhr of Weese [1932], or by a thermoelectric Stromuhr[1] of Rein [1931]. The general blood-pressure and the heart rate of the dog were measured in the femoral artery, the respiration of the animal is also registered.

The results of a typical experiment are shown in the following table:

Carotid sinus pressure	General arterial blood-pressure	Central blood flow per min. (one carotid)	Respiratory rate per min.
165 mm. Hg	60 mm. Hg	200 c.c.	10
20 mm. Hg	180 mm. Hg	450 c.c.	20

Fig. 5 shows a schema of the results of several experiments; a lowering of pressure in the isolated innervated carotid sinus produces a reflex increase of the tonus of the vaso-constrictor centre, with increase of femoral arterial blood-pressure and heart acceleration, but at the same time, one observes a decrease in the femoral blood flow and a considerable increase in the central blood flow and a stimulation of the respiratory centre. Conversely, an increase of the pressure in the isolated innervated carotid sinus brings about a reflex inhibition of the activity of the vaso-constrictor and respiratory centres, although the central blood supply is decreased.

These experiments thus demonstrate that a low carotid sinus pressure produces a reflex increase of the activity of the vaso-motor, cardio-acceleratory and respiratory centres, although at the same time not only the central blood-pressure but also the central blood supply is increased. These experimental facts clearly show once more that the activities of the vaso-motor, cardio-regulatory and respiratory centres are not regulated and influenced directly by the central blood-pressure and blood supply (theories of Gesell, Schmidt and Raab) but indirectly by the reflexes of the carotid sinus, especially by the carotid sinus pressure.

The central blood flow is dependent on the general arterial pressure, the cerebral and medullary blood vessels not participating actively in the general vaso-motor reflex regulation of the circulation, *i.e.* the blood vessels of the central nervous system are "passive." The carotid sinuses are the main reflex regulatory vascular zones for the central blood-pressure and the cerebral blood flow.

In case of a fall in the arterial blood-pressure, the heart frequency, the

[1] The experiments with the Thermostromuhr were performed in the Physiological Institute of Prof. A. K. Noyons, at Utrecht. We wish to express to Prof. Noyons our best thanks.

arterial and venous vaso-motor tonus, the adrenal secretion and the respiration are stimulated and increased by way of the aortic and carotid sinus reflexes; the arterial and venous vaso-motor tonus is increased in the peripheral and splanchnic areas and the blood flow in these areas

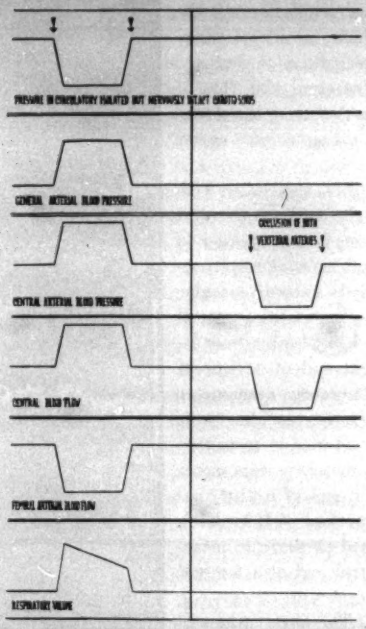

Fig. 5. Influence of the central blood-pressure and the carotid sinus pressure on the general blood-pressure, the central arterial blood-pressure, the central arterial blood flow, the femoral arterial blood flow, the respiratory volume. Dogs prepared following the method of Fig. 4. Up, increase. Down, decrease.

is diminished [C. Heymans, 1929*a*; Rein, 1931], but the blood flow is increased, with the increase of the general blood-pressure, in the intra-central arteries; the arterial blood is thus deflected from the peripheral and splanchnic areas to the central nervous system. In case of an increase of the pressure in the aortic and carotid sinus areas the opposite reactions occur.

II. Carotid sinus reflexes and cerebral vaso-motor tonus.

In order to test our conclusions that the cerebral vessels do not actively participate in the general vaso-motor reflex regulation of the circulation, we have instituted pharmacological investigations to compare the state of the cerebral with that of the muscular vessels during the establishment of carotid sinus vaso-motor reflexes[1].

Method. In dogs anæsthetized with orthochloralose, the cardio-aortic and carotid sinus nerves are cut in order to provoke a general arterial hypertension due especially to a strong vascular constrictor tonus; the vascular arterial tonus is measured in a leg by connecting the peripheral end of a femoral artery with a mercury manometer (method of Nolf), the vascular tonus of the cerebral arteries is measured by connecting a mercury manometer with the cephalic end of an internal carotid artery (method of Hürthle). The general blood-pressure is measured in the central end of a femoral artery.

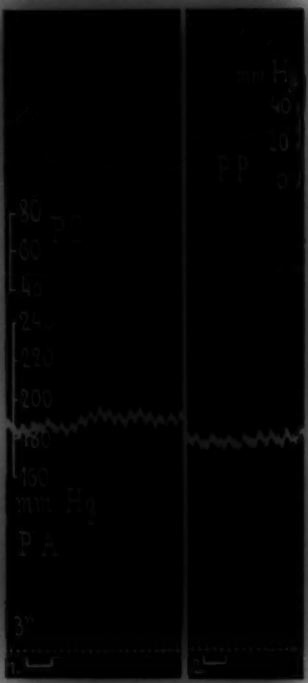

Fig. 6. Dog anæsthetized with chloralosane, aortic and carotid sinus nerves cut. Upper record, vaso-motor tonus of the leg. Middle record, vaso-motor tonus of the cerebral circulation. Lower record, general femoral blood-pressure. At 1, injection into the arterial circulation of the leg of 0·0001 mg. acetylcholine. At 2, injection into the arterial cerebral circulation of 0·002 mg. acetylcholine.

Experiments. The observations of a typical experiment are shown in the curves of Fig. 6. The dog has been prepared as described above. At 1 (Fig. 6) a dose of 0·0001 mg. acetylcholine is injected in the arterial blood stream of the leg, this injection produces an immediate and pronounced relaxation of the arterial peripheral vaso-motor tonus, with a return to the initial vascular tonus after a short

[1] Preliminary report, *C. R. Soc. Biol.*, Paris, 1933, 113, 74.

time. At 2 (Fig. 6) a dose of 0·002 mg. acetylcholine is injected into the blood stream of an internal carotid artery towards the cerebral circulation and, as shown by the curve, no dilatation of the cerebral vessels occurs and also, as shown by the general blood-pressure, no change in the tonus of the vaso-constrictor centre. Similar experiments have been done with different doses of acetylcholine or histamine, and all clearly show that at low carotid sinus pressures, or after section of the carotid sinus and aortic nerves, the muscular and splanchnic vascular areas are contracted, while the cerebral vessels are, on the contrary, not contracted, but passively dilated by the increase of the general blood-pressure.

These pharmacological controls thus confirm our experimental observations upon the central blood flow changes during the carotid sinus vaso-motor reflexes. The cerebral blood vessels are not constricted by the increased vascular tonus in response to a low arterial blood-pressure in the vaso-sensitive vascular areas of the aorta and carotid sinus, and the centres of regulations of heart rate, and the vaso-motor and respiratory centres are not directly sensitive either to changes in central blood-pressure nor to any but extreme changes in central blood supply.

These observations and conclusions do not, however, exclude the possibility of direct sensitivity of the cardio-regulatory, the vaso-motor and respiratory centres to humoral chemical factors. It is established that acute central anoxæmia or asphyxia causes a direct stimulation of the vaso-constrictor centre, with increase of general blood-pressure, and that a respiratory hyperventilation causes, on the contrary, a fall of the general blood-pressure [Dale and Lovatt Evans, 1922]. The same phenomena occur in dogs with denervated aorta and carotid sinus. The section of the four "Blutdruckzügler" produces, by suppression of the reflex vaso-depressor and cardio-inhibitory tonus, a very considerable and chronic arterial hypertension with tachycardia; the vaso-constrictor and cardio-acceleratory tonus remains very high, although the central blood-pressure and the central blood supply are and remain increased, while the CO_2 content of the arterial blood is low [C. Heymans, Bouckaert and Dautrebande, 1931]. But if the arterial CO_2 content is still further decreased, *e.g.* by an excessive artificial pulmonary ventilation, the central vaso-constrictor tonus under these added pathological conditions is now decreased and the general blood-pressure falls (Fig. 6).

We may conclude that the vaso-motor centre maintains a state of vaso-constrictor tonus in consequence of the arterial CO_2 content, but that this tone is mainly depressed and regulated reflexly by the arterial blood-pressure and not directly by the central blood-pressure nor by the

central blood supply; and further, that only very low cerebral pressure and extreme decreases of cerebral blood flow (acute anoxæmia or asphyxia) may directly stimulate the vaso-tonic centres, while a considerable decrease of the CO_2 content of the arterial blood does diminish the tonic activity of the vaso-motor centre.

The experiments described in a recent paper [C. Heymans, Bouckaert and Dautrebande, 1931] have shown that, in agreement with

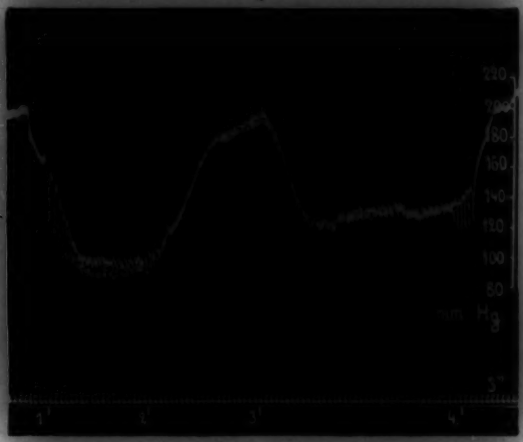

Fig. 7. Dog, anæsthetized with chloralosane; cardio-aortic and carotid sinus nerves cut. Curve of the general femoral blood-pressure. At 1, pulmonary artificial hyperventilation with air. At 2, pulmonary artificial ventilation with air and CO_2. At 3, pulmonary artificial hyperventilation with air. At 4, artificial respiration stopped.

Roberts [1924], anoxæmia has no, or very little, direct stimulatory effect on the respiratory centre; it has also been shown that the respiratory response of the animals to CO_2 inhalation is diminished after denervation of the aorta and carotid sinus. These facts were confirmed recently by the experiments of Schmidt [1932], Selladurai and Wright [1932]. But the respiratory centre certainly is more directly sensitive to the arterial CO_2 content than is the vaso-motor centre. Our experiments show, indeed, that the decrease of central blood-pressure and blood supply, produced by the occlusion of the vertebral arteries or by the occlusion of the efferent branches of the common carotid arteries or

by the occlusion of the carotid arteries after carotid sinus denervation, does not provoke a direct stimulation of the respiratory centre; and on the other hand that the decrease of arterial pressure in the aortic arch or in the carotid sinus does provoke a reflex stimulation of the respiratory centre; these same experiments further show that the reflex changes of respiratory activity, produced by the changes in carotid sinus or aorta pressure, are counteracted after a short time by the humoral factors resulting from the pulmonary hyperventilation. Hence, while the arterial blood-pressure, the tonus of the vaso-motor centre, of the animals remains high during low carotid sinus and aorta pressure or after denervation of the aorta and carotid sinus, the hyperpnœa does not persist.

It may thus be said that the arterial blood-pressure regulates the activity of the respiratory centre especially through the aortic and carotid sinus reflexes; but that the chemical humoral factors have also a reflex as well as an important central regulatory effect on lung ventilation.

Summary.

1. In dogs, the low cerebral blood-pressure, and the reduction of cerebral blood supply produced by occlusion of the efferent branches of the common carotids, by the occlusion of the vertebral arteries, or by the occlusion of the denervated common carotid arteries, do not directly stimulate the vaso-motor, cardio-regulatory, nor the respiratory centres.

2. At low carotid sinus pressure, the vaso-tonic and respiratory centres are stimulated reflexly by way of the carotid sinus nerves, although the cerebral pressure and the cerebral blood flow are both increased by these vaso-motor reflexes.

Conversely, at high carotid sinus pressure, the vaso-tonic and respiratory centres are depressed by the carotid sinus reflexes, although the cerebral blood-pressure and blood supply are at the same time decreased by these circulatory reflexes.

3. The vaso-motor, cardio-regulatory, and respiratory centres are not directly sensitive to the physio-pathological changes in cerebral blood-pressure and in cerebral blood supply, unless these are very extreme.

4. The cerebral blood supply is passively dependent on the general and the cephalic arterial pressure, which is regulated by the aorta and carotid sinus reflexes. In case of decrease of cephalic arterial pressure the blood is deviated by means of the carotid sinus reflexes from the peripheral and splanchnic areas to the cephalic circulation. Conversely, in case of arterial hypertension, the carotid sinus reflexes cause blood to be deviated from the encephalic circulation to the peripheral and splanchnic areas.

5. The arteries in the central nervous system do not participate actively in the "general" reflex regulation of the blood-pressure.

6. The tonus of the arterial vaso-constrictor centre, which is normally in a state of reflex inhibition because of the normal blood-pressure acting on the nerve endings of the aortic and carotid sinus areas, is mainly maintained by the arterial CO_2 tension; this central vaso-constrictor tonus is reduced if the CO_2 content of the arterial blood is much decreased.

7. The respiratory centre is more directly sensitive than the vaso-motor centre, not to anoxæmia but to the CO_2 content of the arterial blood. By low carotid sinus and aorta pressure, or after section of the aorta and carotid sinus nerves, the general blood-pressure increases and remains chronically very high, although the cerebral blood-pressure and the cerebral blood flow are both subnormal, and the CO_2 content of the arterial blood low; but the activity of the respiratory centre which at first is also increased, returns progressively to normal, and later may sometimes be decreased.

8. For the regulation of the blood-pressure, the aorta and carotid sinus nerves are of predominant physiological importance. For the lung ventilation, the aorta and carotid sinus reflexes have also an important, though not so predominant a regulatory function.

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THE ACTION OF VERATRINE, CURARE AND STRYCHNINE ON THE RESPONSE OF MEDULLATED NERVE.

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THERE is a considerable literature relating to the action of veratrine on nerve, but the effects claimed are not very striking. Graham and Gasser [1931], who give various references to previous work, found that the after-potential following a shock is increased and prolonged as the result of treatment with veratrine, and that the effect is made more obvious, either by stimulating for some time or by partial asphyxiation ("40 min. in nitrogen"). According to Schmitt, quoted by Gerard [1932, p. 540], veratrine greatly increases the resting oxygen consumption, and since, according to Hill [1933], a nerve in air may not be far from asphyxiation in its interior owing to its resting metabolism, it may well have been the case, in the experiments by Graham and Gasser, that even at rest, and more particularly when stimulated, their nerves were suffering to some degree from asphyxiation. It has been found, at any rate, that complete asphyxiation has an enormous effect in allowing the veratrine effect in nerve to be manifested, a fact which has led to further experiments on a similar effect of asphyxiation on nerves treated with curare and strychnine, and in some later experiments by Cowan and Ing [1933], with quaternary ammonium salts.

The effect of veratrine found by Graham and Gasser was entirely on the after-potential and not at all on the "spike." An instrument sufficiently rapid to follow the rapid changes of the latter is insufficiently sensitive to record the former except during its earlier stages when it is large. For such reasons a sensitive moving magnet galvanometer was employed. This, with a period of about 1 sec., merely records ballistically the "spike" of the action potential, but the relative shortness of its

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period allows it to follow the after-potential in its later stages with sufficient accuracy. Having a high resistance (20,000 ohms) it was more than sufficiently sensitive.

The nerve (sciatic of Hungarian *R. esc.*) was placed in a special chamber on silver silver-chloride electrodes and stimulated by shocks or a tetanus from a Harvard induction coil. The injury potential was balanced. The sensitivity of the galvanometer was varied as necessary with a shunt. The negative variation of the injury current, to use the classical term, was recorded in response either to a single shock or to a tetanus of about $\frac{1}{2}$ sec. duration, which was sufficiently long to give the maximum response. The end of the nerve was well crushed before laying it on the electrodes. The chamber, which had a glass cover, was kept moist by the slow bubbling of gas through water into it. Hydrogen when used for asphyxiation was previously passed over heated copper to remove traces of oxygen.

Experiments with veratrine.

When the action of veratrine was tested without asphyxiation the nerve was first soaked in Ringer's solution containing veratrine of the required concentration, namely 1/50,000 to 1/100,000 g. per c.c., and then placed in oxygen in the chamber. A stronger solution of veratrine hydrochloride was prepared and neutralized and added in appropriate quantities to the Ringer's solution. With the galvanometer employed no difference at all could be detected between the normal and the veratrinized nerve in respect of the form of the response; following treatment with veratrine the response was perhaps somewhat larger, but if this was due to slightly prolonged after-potential the whole effect was recorded together ballistically with the "spike."

In the experiments with asphyxiation the following procedure was adopted. The nerve was first mounted in its chamber and the galvanometer response recorded to a tetanus or a shock. Then, without moving the nerve, the veratrine Ringer's solution was run in and left for the appropriate time. Then hydrogen was introduced and passed continuously through the chamber from the purifier and the water bottle, the galvanometer reading being recorded at regular intervals in response to stimulation. It was found in control experiment that the operation of introducing and removing Ringer's solution had no effect on the action current. The veratrine concentration in these experiments was generally about 1/30,000 and the solution in the later experiments was buffered by phosphate to *p*H 7·2.

During the asphyxiation with hydrogen the galvanometer response gradually diminished but remained of the same type: there was no sign of any characteristic veratrine effect. This continued until the nerve was completely asphyxiated and no response at all was obtained. When, however, oxygen was admitted to the chamber, the nerve rapidly recovered and within a few minutes showed a completely new type of response. After the usual ballistic deflection corresponding to the "spike" of the action potential, the galvanometer did not return to its base line but remained deflected for a period as long as 10 min. According to the effectiveness of the poisoning and according to the amount of previous stimulation it would follow one or other of the curves shown in Fig. 1.

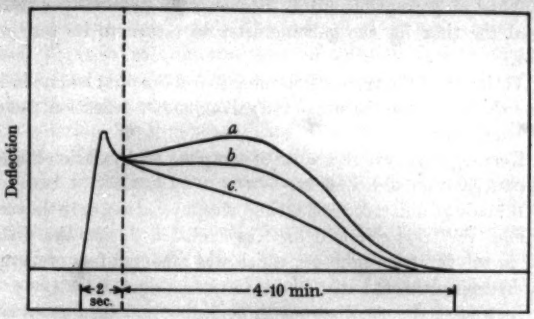

Fig. 1.

This curve, which is almost entirely due to the after-potential, has a form very similar to that of the usual veratrine contracture of striated muscle.

The form of the deflection given by a normal unpoisoned nerve after asphyxiation and recovery in oxygen is of the original form, showing none of the characteristics of the veratrine response. In the earlier stages of recovery the response is somewhat larger than normally.

With the veratrinized nerve the form of the response is the same to shocks or to short tetani. The time required for asphyxiation of normal nerve was about 3½ hours at the temperature of these experiments (about 16° C.) and about 1½ hours for the veratrinized nerve. A poisoned nerve treated by asphyxiation and recovery in this way was always dead next day: an unpoisoned nerve still gave an almost undiminished response.

In order to obtain the full veratrine response in its characteristic form it is necessary to wait for several minutes after stimulation—indeed until

the after-potential produced by the previous stimulus has entirely disappeared. This agrees with Hill's [1933] results in respect of the heat production. It is well known that in muscle the veratrine effect is manifested best after a long period of rest.

Various controls and subsidiary experiments were made.

(1) The strength and character of the effect were not found to depend on the hydrogen-ion concentration, at least between *p*H 4 and 7·2.

(2) A second asphyxiation and recovery scarcely increased the effect at all. The second asphyxiation time was only 40 min. instead of 1½ hours: in normal nerve it was 2½ hours instead of 3½.

(3) Variation of the concentration of veratrine between 1/15,000 and 1/45,000 had no perceptible effect. Higher concentrations (*e.g.* 1/10,000) increased the time for the galvanometer to return to its zero after a stimulus.

(4) Variation of the time of poisoning (0·5–2·5 hours) had no influence either on the form or on the size of the galvanometer deflection, nor on the time of asphyxiation.

(5) Keeping the nerve in a state of complete asphyxiation for as long as 1½ hours gave no added effect.

(6) It made no difference to the time of asphyxiation, or to the strength of the final veratrine effect, if air was excluded during the preceding soaking in the veratrine solution, which was rendered free of oxygen by passing hydrogen through it.

(7) Instead of the usual sequence, poisoning and then asphyxiation, in two experiments the nerve was first nearly completely asphyxiated and then, without admitting air, an oxygen-free veratrine solution was admitted to the paralysed nerve. The nerve was kept for 1 hour in the oxygen-free solution, which was then removed without admission of air. During the recovery process the characteristic phenomena of veratrine poisoning appeared just as in the usual experiments.

(8) A nerve which before poisoning had already undergone asphyxiation and recovery showed a reaction to veratrine poisoning exactly the same as a normal nerve.

(9) Asphyxiation with carbon dioxide had just the same effect on the veratrinized nerve as asphyxiation with hydrogen or nitrogen.

All the experiments and controls suggest that before asphyxiation veratrine is unable to penetrate to the interior of the nerve fibre, but is held by solution or adsorption either in the interspaces between the fibres or in their sheaths. During a state of altered permeability produced by asphyxiation the drug is able to penetrate and cause its characteristic effect.

Experiments on conduction in veratrinized nerve.

If the nerve of a muscle-nerve preparation be poisoned with veratrine at its central end far from the muscle and excited in the veratrinized part, how will the muscle connected to the unpoisoned end react? If the veratrine response were a sequence of impulses set up by a single shock, then it should be transmitted to the normal part of the nerve and the muscle should respond with a tetanus. If, on the other hand, the veratrine effect is of the nature of a prolonged after-potential, the muscle might be expected to respond by a single twitch to a shock applied in the distant veratrinized part.

A frog's gastrocnemius-sciatic preparation was hung in a closed glass tube with its nerve downwards. The free end of the nerve was held with a thread. Veratrine solution was then run upwards so as to immerse the lower end of the nerve for a distance of 2 cm. or more, up to three-quarters of its length. In some experiments still more of the nerve was covered with the solution, but it appeared that even when this was very carefully done the veratrine was able to reach the muscle by diffusion along the nerve, which at the end gave a veratrine response on direct stimulation. The concentration of the veratrine solution was between 1/10,000 and 1/30,000 in phosphate Ringer solution at *p*H 7·2. The time of immersion of the nerve was about 1¼ hours. The veratrine solution was then withdrawn and the air replaced by oxygen-free hydrogen. After asphyxiation for 4 hours and recovery for 15–30 min. the nerve-muscle preparation was placed in a suitable chamber and the response of the muscle to a shock applied to the nerve recorded on smoked paper. First of all the poisoned end of the nerve was stimulated, then the nerve completely cut away and the muscle stimulated directly in order to control whether the poison had reached the muscle by diffusion. The nerve which had been cut off was then placed in a chamber for measuring the action current, so that stimulating and galvanometer electrodes both lay on the poisoned portion, and observation of the action current showed whether the nerve had in fact been properly veratrinized.

With care half to two-thirds of the nerve can be veratrinized without any of the poison reaching the muscle by diffusion. When such a nerve-muscle preparation is stimulated at the poisoned end the muscle responds by an ordinary single twitch although the action current of the nerve itself in the poisoned region shows the characteristic veratrine effect. If sufficient care is not exercised, for example if the veratrine solution gets splashed on the muscle, or if the nerve is too deep in the solution and the

veratrine can reach the muscle by diffusion, the muscular contraction shows a veratrine effect when the nerve is stimulated, but the same effect appears when the muscle is stimulated directly after cutting the nerve away.

It appears, therefore, that the veratrine response of a nerve produces nothing but a single twitch in a muscle to which it is connected through a piece of unpoisoned nerve, so that the veratrine effect in nerve is not due to repetitive response to a single shock.

Experiments with curare.

The effect of asphyxiation and recovery in allowing veratrine to produce its characteristic effect on nerve suggested that similar experiments should be made with curare and strychnine.

Curare was employed in a concentration of 1 mg. per c.c. of Ringer's solution at *p*H 7·2. 2·5 mg. of the same curare injected into a Hungarian frog of weight about 50 g. produced paralysis within about 7 min. A gastrocnemius-sciatic preparation of a Hungarian frog placed in the 1/1000 solution was paralysed in 80 min. With a 1/10,000 solution paralysis was complete in 120 min. In the experiments, therefore, a nerve-muscle preparation of a Hungarian frog was left for 2 hours in the 1/1000 solution and then tested to see that the curare had produced its characteristic paralysing effect. Then the nerve was cut away from the muscle and treated in the same manner as in the veratrine experiments described above. For a control the opposite muscle-nerve preparation and nerve were treated in the identical manner but without curare. Both nerves then had their action currents measured after asphyxiation and recovery in oxygen.

After poisoning without asphyxiation the curarized nerve showed no essential difference from the normal nerve except that the deflection produced by the action current was perhaps somewhat greater. After asphyxiation, however, on readmitting oxygen a considerable difference appeared. In the normal nerve, within half an hour, the deflection had reached its normal value. The curarized nerve, on the other hand, recovered only very slowly and even in 1½–2 hours the deflection recording its action current was still only a fraction of the original value. There were signs of a small long-continued after-potential.

Curare, therefore, has little or no effect on a medullated nerve until the nerve is asphyxiated. Then its harmful poisonous influence is manifested by a large reduction in the action potential. The experiments were

not as decisive as they might have been, owing perhaps to the size of the curare molecule and its difficulty in penetrating the fibres. Recent experiments, however, by Cowan and Ing [1933] have shown that quaternary ammonium salts, which have a curare-like action, while producing no detectable effect on normal unanæsthetized nerve, are found completely to have destroyed its activity when oxygen is re-admitted to it after asphyxiation. These substances also are apparently unable to penetrate the nerve sheath in the ordinary way, but during prolonged oxygen want are able to penetrate, so that oxygen cannot cause any restoration after asphyxiation.

Experiments with strychnine.

The experiments with strychnine were similar to those with curare. A gastrocnemius-sciatic preparation soaked in Ringer's solution containing strychnine hydrochloride 1 mg. per c.c. at *p*H 7·2 was found to be completely paralysed in 125 min. For the chief experiments a nerve-muscle preparation was soaked in the veratrine solution for 2½ hours and then tested for complete paralysis by stimulation.

After poisoning the deflection due to the action current was either normal or showed a slight after-potential. If the poisoned nerve was kept in the chamber for several hours the galvanometer deflection either remained constant or diminished slightly and gradually. Asphyxiation had the same effect as in the curare experiments. After complete disappearance of the action potential during asphyxia, when oxygen was readmitted the deflection returned only slowly and even then only to a fraction of what it was before it began. Such deflection as there was showed signs of a continued after-potential.

The effects of curare and strychnine are so similar that without previous knowledge it would not be possible to say whether the effects were produced by the one or the other.

Summary.

Medullated nerves (frog's sciatic) are unaffected or only slightly affected by veratrine, curare and strychnine in concentrations sufficient to cause the characteristic effects on muscle-nerve preparations. The drug presumably remains either in solution in the fluids between the fibres or adsorbed by their sheaths. When, however, a nerve is completely asphyxiated in hydrogen these three substances are able to penetrate, and when oxygen is readmitted the characteristic effects are found. The veratrinized

nerve after asphyxiation and recovery shows a prolonged after-potential of considerable magnitude persisting for many minutes. Nerves treated with curare and strychnine have their response to stimulation greatly reduced.

When the central end of the nerve of a muscle-nerve preparation is veratrinized by soaking in the drug, followed by asphyxiation and recovery, the muscle attached to the nerve shows a normal single twitch in response to a shock to the poisoned section of the nerve, although the nerve itself gives a characteristic veratrine effect. Presumably, therefore, the veratrine effect is not due to a repetitive transmissible response to a single shock.

I wish to express my sincere thanks to Prof. A. V. Hill for his suggestion of the present work and for his advice and encouragement throughout it. To Mr J. L. Parkinson I am indebted for much advice and assistance.

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THE EFFECT OF QUATERNARY AMMONIUM SALTS UPON NERVE.

(PRELIMINARY COMMUNICATION.)

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IT is well known that quaternary ammonium salts exert a curare-like action, blocking the passage of excitation from nerve to voluntary muscle; these curariform properties were discovered by Crum Brown and Fraser [1869] and have been confirmed by many other workers. Ing and Wright [1931] made quantitative measurements of the activities of a number of these salts at different concentrations (0·25–2·0 millimols per litre) by means of frog's nerve-sartorius preparations and showed that the paralysis was reversible. Boehm [1910], and Höber and Waldenberg [1909] established that, in higher concentrations than those required to produce "curarization," these salts reduced the response of muscle to direct stimulation, and Jodlbaur [1900] showed that high concentrations (9–18 millimols per litre) of tetramethyl-ammonium chloride diminished conduction in nerve.

EXPERIMENTS ON NON-MEDULLATED NERVE.

During September, 1932, some experiments were made by one of us (S.L.C.) at Plymouth with quaternary ammonium salts upon nerves from the walking legs of the spider crab (*Maia squinado*). The nerve was dissected in the manner described by Levin [1927] and allowed to rest in aerated sea water for an hour; it was then mounted in a paraffin wax chamber, the peripheral end crushed and the chamber closed with a glass cover. The action current was led off from the nerve by strips of filter paper, moistened with sea water, to calomel electrodes, which had been made up with sea water, and thence to a high resistance galvanometer (Cambridge A and M: sensitivity 1 mm. = 4.10^{-10} amp. at 3 metres and

[1] Working with a grant from the Medical Research Council.

period 3·8 sec.) which was critically damped. The galvanometer spot was brought to a convenient position on the scale by partially balancing the "injury potential" of the nerve by an applied E.M.F. from a rotary potential divider. Just maximal tetani [Beresina and Feng, 1933], passing through a shunt of 1500 ohms and a series resistance of 3000 ohms to render the condenser (0·95 μF) discharge time approximately independent of the resistance of the nerve, were provided by a rotating commutator [A. V. Hill, 1932]. The stimulus was applied by means of silver silver-chloride electrodes which were about 7 cm. from the nearer action current lead and interposed between the two sets of electrodes was another silver silver-chloride electrode connected to earth to prevent leakage of stimulus into the galvanometer circuit. A nerve in good condition gave 100–150 mm. deflection for a 15 sec. tetanus—frequency 49 per sec.; after testing, it was placed in sea water containing added tetramethylammonium chloride for 1 min., remounted and tested again. A concentration of 50 millimols per litre killed the nerve immediately, rendering it white and opaque: concentrations of 10 millimols and 0·9 millimol per litre rendered the nerve inexcitable without doing any visible damage or impairing the oxidative mechanism for maintaining the "injury potential" [Furusawa, 1929]. The same concentrations of tetraethylammonium chloride produced similar effects.

Tetramethyl- and tetraethylammonium salts differ widely in their curariform properties on frog's nerve-muscle preparations, the latter being not only many times weaker than the former, but also reducing the response of the muscle to direct stimulation at all concentrations at which it achieves paralysis [Marshall, 1914; Ing and Wright, 1931]. The fact that no difference was observed between the actions of these salts on crab's nerve may mean that 1 millimol per litre is far too high a concentration for the study of the effects of quaternary ammonium salts on these sensitive tissues. Experiments with tetraethylammonium iodide on frog's nerve, described below, support this view. The sensitiveness of crab's nerve is well illustrated by the action of veratrine which depresses the action current in concentrations greater than 1 in 20,000 but greatly increases it in concentrations of 1 in 10 to 100 millions [Cowan, 1933].

Experiments on medullated nerve.

In view of the results just described and of the experiments of Fromherz described in the preceding communication it was thought of interest to see whether medullated nerve when asphyxiated would become permeable to quaternary salts.

Materials.

The work was done with a batch of large Hungarian frogs (*R. esc.*) which had been at the laboratory since the beginning of January. Four quaternary ammonium salts were used: tetramethylammonium iodide, the simplest member of the group; octyltrimethylammonium iodide, which has been widely used in curarization experiments [Nachmansohn, 1929]; strychnine methiodide, which appears to be a more active "curarizing" compound than any of the simple quaternary ammonium salts (H.R.I., unpublished result); tetraethylammonium iodide, which differs radically from the other three salts in curariform properties, was also used in preliminary experiments. Recrystallized specimens of the quaternary ammonium iodides were prepared by one of us (H.R.I.).

In a few early experiments nitrogen freed from oxygen by passage through alkaline hydrosulphite [Kautsky and Thiele, 1926] was used, but in most it was found simpler to use hydrogen which had been freed from oxygen by passage over red hot copper gauze.

Apparatus.

Thick-walled wax chambers, closed by glass plates sealed on with plasticine and provided with gas inlets and outlets, were employed. The action current was led off from the nerve by filter paper strips soaked in Ringer's solution to calomel electrodes, also made up with Ringer's solution and thence to the galvanometer already mentioned. Maximal tetani, with 1500 ohms shunt and 4500 ohms series resistance, were provided by condensers (0·2 μF) charged to 6 or 8 volts and the rotating commutator [A. V. Hill, 1932] and applied to the nerve through silver silver-chloride electrodes. This time the distance between the stimulating electrodes and the nearer action current lead was only 3 cm.; however, an earthed silver silver-chloride electrode interposed between the stimulating and action current electrodes obviated leakage.

Experimental.

Two gastrocnemius-sciatic preparations were made and allowed to rest in phosphate buffered Ringer's solution for a quarter of an hour. They were then taken out and tested by indirect stimulation, transferred to phosphate Ringer containing the added quaternary ammonium iodide; subsequently they were taken out and tested by indirect stimulation about every quarter of an hour until response had disappeared and then left in a renewed solution for half an hour longer. At the end of that period one preparation was tested again, the nerve was cut off, mounted,

ligatured between the action current leads and the peripheral end crushed. Tetani of 20 sec. duration, frequency 85 per sec., were applied every 1½ min. until the initial treppe was over [Gerard, 1927] and a constant response was obtained. This was usually about 25 mm.; the position of the galvanometer spot could be read to 0·1 mm. Nitrogen or hydrogen was turned on and the chamber was sealed until it would maintain an excess pressure of 15 mm. of water with gas passing at the rate of about ½ c.c. per sec. During the period of asphyxiation the action current was tested about every quarter of an hour; a 20 sec. tetanus was given to get rid of any treppe effect and the mean of the responses to two more 20 sec. tetani at 1½ min. intervals was taken to be the response at that time. The room temperature was maintained at 19° C.; usually the action current vanished in 6 hours and asphyxiation was continued for a further hour. When oxygen was admitted the "injury potential," which had fallen by about 50 p.c., rose again towards its former value and after 5–10 min. the action current was examined; further readings were taken at 3–5 min. intervals for half an hour, subsequently every quarter of an hour during the succeeding 2 hours. In some experiments the "crush sealing over effect" was very evident and the response became diphasic [Gerard, 1930], and consequently at the end of each experiment the peripheral end of the nerve was re-crushed to make certain that the action current had really vanished; also, it was noted that the "injury potential" was undiminished.

Finally the other gastrocnemius-sciatic preparation, which had been resting in oxygenated Ringer's solution containing the quaternary ammonium iodide, was tested and found to be still "curarized." The nerve was cut off, mounted as already described and stimulated by groups of three 20 sec. tetani at 1½ min. intervals. These stimuli were repeated every 5 min. for 20 min. In all experiments, except some with tetraethylammonium iodide, the magnitude of the response of the control nerve was within 10 p.c. of that which the asphyxiated nerve had given initially.

In a few instances at the end of an experiment the nerves were put into oxygenated phosphate Ringer and left overnight. At the end of 15 hours no recovery was observed in the nerve which had been asphyxiated after poisoning, whilst the nerve which had only been poisoned gave approximately two-thirds of its former action current.

Results and discussion.

When oxygen was admitted, after asphyxiation, no recovery of action current occurred during 3 hours if a concentration of 2 millimols per litre of octyltrimethylammonium iodide had been used (Fig. 1 *A*); the injury

potential rose nearly to the value exhibited at the beginning of the experiment and consequently the oxidative mechanism whereby it is normally maintained [Furusawa, 1929 and 1931] was unimpaired. Half this dose of the same drug (Fig. 1 *B*) permitted the action current to rise to about 140 p.c. of its initial value, but recovery was only transient and disappeared in 1½ hours. Two millimols per litre of tetramethylammonium iodide were found rather less effective than 1 millimol per litre of octyltrimethylammonium iodide, the temporary recovery of action current

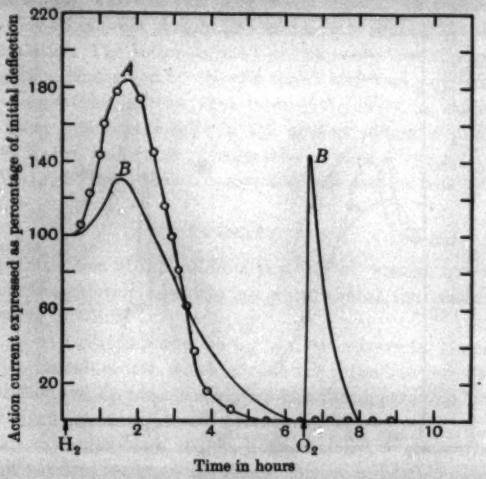

Fig. 1. The effects of two different concentrations of octyltrimethylammonium iodide on the action current in frog's nerve during and after asphyxiation at 19° C. *A*, 2 millimols per litre; *B*, 1 millimol per litre. In experiment *B* room temperature accidentally rose at 6 hours to 23° C. and was maintained at that level until oxygen was admitted to the nerve and then restored to 19° C.: to compensate for the greater metabolism at the higher temperature the "no action current period" was reduced from 1 hour to 25 min.

lasting somewhat longer (Fig. 2 *A*). Strychnine methiodide in a concentration of 1 millimol per litre was quite as effective as 2 millimols per litre of octyltrimethylammonium iodide in causing the action current to disappear (Fig. 2 *B*).

Fig. 2 *C* gives the curve for a control nerve which was merely soaked in phosphate buffered Ringer's solution without poisoning, asphyxiated and allowed to recover in oxygen.

The magnitude of the rise in action current during the first two hours in hydrogen [cf. Gerard, 1930] varied a good deal in different control nerves and probably the variations between different poisoned nerves, shown in Figs. 1 and 2, are of no significance. When oxygen was admitted to a lightly poisoned nerve the rise of action current, about 140 p.c., was not as large as that in a control nerve, more than 200 p.c. This would indicate either that some of the fibres never recovered their excitability or that recovery was incomplete in most of the fibres.

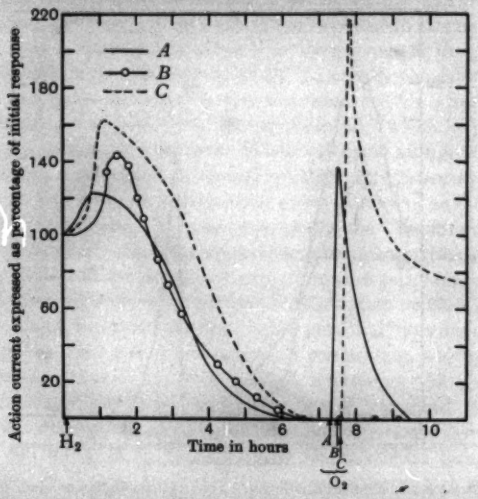

Fig. 2. The action current in frog's nerve during and following asphyxiation in hydrogen at 19° C. *A*, after poisoning with 2 millimols per litre of tetramethylammonium iodide; *B*, after poisoning with 1 millimol per litre of strychnine methiodide; *C*, control after soaking for 1½ hours in phosphate buffered Ringer's solution.

These results suggest that the curariform properties of quaternary ammonium salts may be due to their rendering the non-medullated endings of the motor nerves inexcitable if these endings are assumed to be permeable to the quaternary cations without previous asphyxiation.

A few experiments were made with tetraethylammonium iodide in a concentration of .10 millimols per litre. The muscles of the two preparations showed spontaneous twitching and although paralysis to indirect

stimulation was incomplete at the end of 2 hours, one of the nerves was cut off and mounted. Usually the action current was twice as large as that given by a normal unpoisoned nerve. After asphyxiation was over and oxygen had been admitted the poisoned nerve showed a rather exaggerated supernormal phase in the recovery of action current, then the response fell to 25 p.c. above its initial value and remained steady for about 1½ hours. The control preparation, which had been soaking in oxygenated phosphate buffered Ringer containing the added tetraethylammonium iodide, was still twitching when tested: the muscle gave a small response to indirect stimulation and only a slightly larger one to direct stimulation. The action current of this nerve was approximately 25 p.c. less than that given by the one which had been asphyxiated.

These observations indicate that tetraethylammonium iodide differs from the other quaternary salts in the kind of action it produces in asphyxiated nerve, and are very suggestive in view of the conclusion of Ing and Wright [1931] that this salt does not exert a true curariform action.

Summary.

1. Concentrations of 0·9 millimol per litre of tetramethyl or tetraethylammonium chloride added to sea water abolish the action current in crab's nerve in 1 min.

2. Frog's medullated nerve, on asphyxiation, becomes permeable to quaternary ammonium ions which abolish the action current when used in concentrations which readily achieve a curariform paralysis but do not ordinarily affect either muscle or nerve.

3. Tetraethylammonium iodide is anomalous; a concentration of 10 millimols per litre increases the action current in frog's nerve.

Our sincere thanks are due to Prof. A. V. Hill for his continued interest in this work. One of us wishes to thank Dr E. J. Allen, Director of the Marine Biological Laboratory, Plymouth, for permission to work there and for all the courtesies extended to him. Also, we are indebted to the Government Grant Committee of the Royal Society and to the University of London, Thomas-Smythe-Hughes and Beaverbrook Funds, for grants to one of us (S.L.C.) towards the cost of apparatus.

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THE REACTIVITY AND ACTIVITY OF THE HUMAN UTERUS AT VARIOUS STAGES OF PREGNANCY AND AT PARTURITION.

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In a previous paper [Robson, 1933] it was shown that the reactivity of the rabbit's uterus to a preparation of the posterior pituitary lobe hormones "pituitrin" during pregnancy showed certain definite variations. During the first half of gestation neither oxytocin nor vaso-pressin caused any contraction of the uterine muscle. This was followed by a stage in which the muscle was affected by vaso-pressin but not by oxytocin. Later on the muscular reactivity to the oxytocic factor returned, gradually increased as pregnancy advanced, and parturition was marked by a very high degree of reactivity. As it has been accepted by a number of investigators that the action of oxytocin on the reactive uterus plays an important part in determining the onset of labour, it appeared of interest to determine the changes in reactivity of the human uterus at the various stages of gestation; the spontaneous rhythmic activity of the muscle was, at the same time, studied.

Methods.

All the human uterine material used in these investigations was removed during operations, and placed at my disposal by the courtesy of Prof. Johnstone, Dr Young, Prof. Hendry, and their assistants, to all of whom I am very greatly indebted. The operations were performed for a variety of reasons, given in the appropriate table (I). The material may be grouped thus: (1) Uterine tissue removed in Edinburgh. This was placed in iced Ringer-Locke solution immediately after removal and transferred to the warm solution $\frac{1}{4}$–$\frac{1}{2}$ hour later. (2) Material removed in Glasgow. This was also placed in iced Ringer-Locke immediately after removal, but several hours elapsed before it was transferred to the warm oxygenated solution. In the majority of cases the muscular tissue has been removed from the anterior wall of the body of the uterus and usually in

a longitudinal direction, but in a few cases the strips were from the lower uterine segment when a lower uterine Cæsarean section had been performed.

An attempt was made in all cases to dissect off strips of muscle of approximately the same length, and containing more or less parallel fibres. These strips were suspended in oxygenated Ringer-Locke solution (in 100 c.c. containers) at 37° C. Records were taken on smoked drums. The standard preparations from the posterior lobe which were used were "pituitrin," "pitocin" and "pitressin," kindly supplied by Parke, Davis and Co.

Results.

As it had previously been found that the rabbit's uterus was not made to contract even by large doses of "pituitrin" in the earlier stages of pregnancy, it appeared possible that a similar condition would be found

Fig. 1. Reactions of uterine strip (H 40) removed at the 8th week of pregnancy. A. 0·1 unit of pitocin added to bath. No effect. B. 0·4 unit of pitocin added to bath. No effect. C. 0·5 unit of pitressin added to bath. D. 0·5 unit of pitocin added to bath. No effect. E. 0·5 unit of pitocin added to bath. No effect. F. 0·01 mg. of histamine added to bath. *S* = Solution changed. Time intervals = 1 min.

in the human uterus. It soon became apparent, however, that the earliest material obtained would react to fairly large doses of pituitrin. For example, muscle removed from a human uterus 8 weeks pregnant (H 40) contracted on the addition of 0·5 unit of pitressin but was unaffected by 1 unit of pitocin. It was thus evident that the reactivity even in the earlier stages would have to be measured more accurately, and this was done by determining the smallest dose of drug added to the 100 c.c. containers, necessary to bring about a motor effect. The same procedure was, of course, adopted for those strips removed at a later stage of gestation. In

the majority of cases the motor effect included a rise in the base line, though in a few experiments where small doses were being used the chief effect consisted in an increase in the height of the rhythmic contractions. The experiments were all carried out for prolonged periods, as it was found that occasionally the maximal reactivity was not immediately exhibited. The experiments were all carried out in duplicate, and the reactivity given for each experiment represents that of the strip which responded to the smaller dose.

The results are tabulated in Table I. When material obtained early in gestation was investigated it was found in certain instances that the muscle was more reactive to vaso-pressin than to oxytocin. Thus, for

TABLE I. To show the reactivity of the uterus at various stages of pregnancy.

Exp. No. H	Duration of pregnancy weeks	Minimum effective dose of drug: Pitocin units	Ergotoxin mg.	Pitressin units	Histamine mg.	Pituitrin units	Source	Remarks
40	8	>1	>0·1	0·5	<0·01	—	E	Albuminuria
4	12	>2	—	2	—	1	E	Chronic nephritis
8	12	2	—	1	—	—	E	—
18	12	0·5	>0·08	0·5	>0·08	—	E	Fibroid
23	12	0·10	—	—	<0·01	—	G	Nervous instability
3	14	—	—	—	—	5	E	No detailed dosage investigation
27	14	>0·6	>0·1	0·5	0·1	—	G	Cardiac disease
36	17	0·05	>0·1	—	—	—	G	Pulmonary T.B.
30	24	0·2	>0·1	0·2	0·004	—	E	Nephritis
2	25	—	—	—	—	5	E	No detailed dosage investigated
1	26	—	—	—	—	5	E	No detailed dosage investigated
5	30	<2	—	<2	—	—	E	Minimal doses not determined
31	30	0·005	0·005	—	—	—	E	Auricular fibrillation
34	30	0·05	—	—	—	—	E	—
32	33	0·015	0·04	—	—	—	E	A.P.H.
22	34	0·1	>0·05	—	>0·03	—	G	Chronic abortions
19	35	0·04	—	—	—	—	G	Placenta prævia
28	36½	0·1	>0·1	—	—	—	G	Contracted pelvis
11	37	0·02	—	—	—	—	E	Toxæmia
25	37	0·01	0·005	—	0·004	—	G	Placenta prævia
21	39½	0·03	>0·08	—	0·004	—	E	—
33	40	0·01	>0·07	—	—	—	E	Acute anteflexion
12	L	0·01	—	—	—	—	G	Contracted pelvis
13	L	0·005	—	—	0·002	—	G	Contracted pelvis
14	L	0·01	<0·01	—	0·01	—	G	Contracted pelvis
17	L	0·01	0·0075	—	0·001	—	G	Placenta prævia
35	L	0·003	0·003	—	—	—	E	Contracted pelvis
15	L	0·05	—	—	—	—	G	L.U.S.
16	L	0·05	—	—	—	—	G	L.U.S. Contracted pelvis
20	L	0·05	<0·01	—	0·004	—	G	L.U.S. Contracted pelvis
29	L	0·005	—	—	0·004	—	E	L.U.S. Contracted pelvis

E = Edinburgh. G = Glasgow. L.U.S. = Lower uterine segment. L = Labour.

example, the strip removed at the 12th week of pregnancy (H 4) was made to contract by the addition of 2 units of pitressin to the bath, while a similar dose of pitocin was without effect. In another case (H 27) a strip removed at the 14th week of gestation reacted to a dose of 0·5 unit of pitressin, while 0·6 unit of pitocin had no effect. The muscle removed in the early stages of pregnancy showed, as a rule, a comparatively low reactivity to oxytocin, though in one case (H 36) the addition of 0·05 unit to the bath was sufficient to bring about a motor effect.

The strips removed between the 17th and 30th weeks of pregnancy showed on the whole a rather more marked reactivity to oxytocin than those removed earlier. Thus in no case was there exhibited a complete lack of reactivity to large doses of oxytocin (as was found in the earlier stages in H 4, 8, 27 and 40), and moreover a high reactivity was observed in one case (H 31) where the strips were removed at the 30th week of pregnancy from a case of auricular fibrillation.

In eight cases muscular tissue was removed between the 33rd and 40th weeks of gestation. The range of reactivity exhibited during this period varied from 0·1 unit to 0·01 unit; the muscular reactivity at this stage is thus greater than that exhibited during the previous periods, although fairly large variations amongst the cases were again observed.

In five cases strips were removed from the body of the uterus actually during labour, and in all cases the dose of oxytocin necessary to cause a motor effect was comparatively small. In three out of five cases 0·01 unit was the minimum effective dose, while in one case (H 35) the addition of 0·003 unit of oxytocin to the bath was sufficient to bring about a motor effect.

In those cases in which a lower uterine segment operation was performed during labour, muscular tissue was removed from that part of the uterus. The reactivity to oxytocin of these strips appears to be significantly less than that of similar strips removed from the body of the uterus during parturition, for in three out of four cases the addition of 0·05 unit to the bath was necessary to elicit a motor effect, the other case reacting to the smaller dose of 0·005 unit.

Reactivity to histamine.

In a number of cases the reactivity of the strips to histamine was also investigated by determining the minimum dose of the drug necessary to cause a motor effect. All the strips removed during parturition showed a high reactivity, a motor effect being produced by concentrations between

1 in 100,000,000 to 1 in 10,000,000; high reactivities were also observed during the various stages of pregnancy investigated, though in several cases (H 13, 22, 27) the effective dose of histamine was considerably higher than at parturition. There appears, however, to be no correlation between the reactivity of the muscle to oxytocin and to histamine, nor does the reactivity to the latter appear to show any increase during the course of gestation.

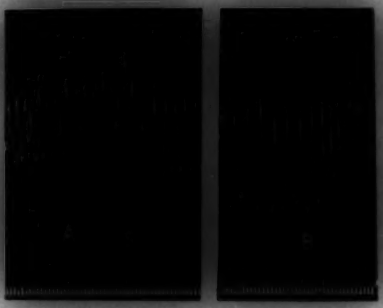

Fig. 2.

Fig. 3.

Fig. 2. Reactions of uterine strip (H 35) removed at parturition. A. 0·003 unit of pitocin added to bath. B. First signal: 0·001 mg. of ergotoxin added to bath. Second signal 0·002 mg. of ergotoxin added to bath. *S* = Solution changed. Time intervals = 1 min.

Fig. 3. Reactions of uterine strip (H 25) removed at the 37th week of gestation. First signal: 0·0025 mg. of ergotoxin added to bath. Second signal: 0·0025 mg. of ergotoxin added to bath. Third signal: Solution changed. Time intervals = 1 min.

Reactivity to ergotoxin.

It is well known that the uterus at parturition is particularly sensitive to the action of ergot, and it appeared of interest to determine whether any definite changes could be found in the reactivity of the isolated strips to an active principle of that drug, ergotoxin. The results are included in Table I and appear to be highly interesting. In the first place the strips removed during parturition all showed a high reactivity to ergotoxin, the muscle responding in every case to concentrations of less than 1 in 10,000,000, and in one case (H 35) the addition of 0·003 mg. of the drug to 100 c.c. of solution was sufficient to cause a contraction. Certain

peculiarities of the reaction to ergotoxin require comment. The minimum effective dose of the drug usually gives quite a marked effect including a rise in the base line. This is different from the reaction to oxytocin, for with the latter substance the reaction to a threshold dose is usually small and may affect chiefly the height of the spontaneous rhythmic contractions. Moreover, the reaction to ergotoxin is markedly sustained and is usually maintained for a long period, even after a change of solution. This type of reaction is illustrated in Fig. 3, in which the action of 0·005 mg. of ergotoxin on a strip of uterus removed at the 37th week of pregnancy (H 25) is shown. In the second place the uterine muscle removed before parturition showed, with three exceptions, not the slightest reaction to ergotoxin (within the limits of the doses used). Three facts are noteworthy among these findings: (1) In those three cases where the muscle removed during pregnancy did react to ergotoxin the reactivity to oxytocin was also high (H 31 gave a response with 0·01 unit of oxytocin and 0·005 mg. of ergotoxin; H 25 with 0·01 unit of oxytocin and 0·005 mg. of ergotoxin; and H 35 with 0·015 unit of oxytocin and 0·04 mg. of ergotoxin). (2) All those strips that were removed before the 30th week of gestation and tested for their reactivity to ergotoxin, namely H 18, 27, 36, 30, showed no reaction. (3) Strips removed at the end of gestation but before parturition (H 21 and 33), though they showed quite a high reactivity to oxytocin, did not respond to ergotoxin.

Spontaneous rhythmic activity.

In all the experiments an attempt was made to estimate the degree of spontaneous rhythmic activity of the isolated strips and the results are presented in Table II; where the strips showed very little or no activity, the *minus* sign has been put in the appropriate column while increasing degrees of rhythmic activities are represented by an increasing number of *plus* signs. The same method of estimating spontaneous activity has been used in these experiments as in the previous investigation reported on the rabbit [Robson, 1933], and, moreover, the length of the strips of human and rabbit muscle used in both series of experiments was substantially the same. When the duplicate strips used in any one experiment showed the same degree of rhythmic activity only this one value is given in the table, but where there was a difference between the two strips, both values are given.

It will, in the first place, be seen (Table II) that no stage of pregnancy investigated is characterized by any definite degree of rhythmic activity of the isolated muscle strips, both high and low rhythmic activity being

TABLE II. Relating the spontaneous activity and reactivity to oxytocin at various stages of pregnancy.

Exp. No. H	Duration of pregnancy weeks	Minimum effective dose of oxytocin units	Spontaneous activity	Remarks
35	Parturition	0·003	+ + + + +	—
13	,,	0·005	+ + + +	—
31	30	0·005	+	—
29	Parturition	0·005	+ +	Lower uterine segment
17	,,	0·01	+ + + + + +	—
14	,,	0·01	+ + + + + +	—
12	,,	0·01	+	—
33	40	0·01	+	—
25	37	0·01	+ + + +	—
32	33	0·015	−	—
11	37	0·02	+ + + + + + +	—
21	39½	0·03	+	—
19	35	0·04	+ + + +	—
20	Parturition	0·05	+ + + + + +	Lower uterine segment
16	,,	0·05	+ + + +	,, ,,
15	,,	0·05	+	,, ,,
36	17	0·05	+ + +	—
34	30	0·05	+ + +	—
28	36½	0·1	+ + +	—
22	34	0·1	+ + + + +	—
23	12	0·1	+ + + + +	—
30	24	0·2	+	—
18	12	0·5	−	—
27	14	0·6	+ + + + +	—
5	30	2·0	+ + +	—
40	8	1·0	+ +	—
8	12	2·0	+ + + +	—
4	12	2·0	+ + +	—

observed in the early stages of gestation as later on; nor did the muscle strips removed during parturition always show marked spontaneous contractions. Secondly, there appears to be no correlation between the rhythmic activity of the strips and their reactivity to oxytocin; for a high degree of spontaneous activity may be associated with a very low reactivity to oxytocin (*e.g.* H 27, 4) and, on the other hand, strips showing only small contractions may respond to small doses of oxytocin (*e.g.* H 31). Moreover, strips removed from the uterus at the same time may show marked differences in their spontaneous activity although their reactivity is substantially the same.

DISCUSSION.

As the uterine muscle used in this investigation was removed at operations for a variety of conditions it cannot be said to represent a completely physiological material; nevertheless, in most cases it seems probable that the factor which made operative interference desirable did

not exert any significant effect upon the uterus. This applies especially to the cases of contracted pelvis, and probably also to a majority of the other cases; and only in those conditions which were likely to interfere with a pregnancy (*e.g.* placenta prævia, hæmorrhage) is it at all probable that any effect on the uterine reactivity may have resulted. It seems therefore valid to draw a conclusion from the data obtained.

An examination of these data at the various stages of pregnancy shows in the first place that quite appreciable differences in reactivity may be observed within a limited period of gestation, these differences being outside the limits of experimental error. It appears possible that at one particular stage of pregnancy different uteri may not exhibit the same reactivity and also that different strips even from one particular region of the same uterus may not necessarily react to the same dose of oxytocin. Moreover the differences observed at parturition between the muscle removed from upper and lower uterine segments strongly suggests that the reactivity of the whole organ is by no means uniform.

A consideration of the average reactivity at several stages of gestation shows, however, that as pregnancy advances the dose of oxytocin necessary to cause a contraction of the muscle gradually decreases; thus to take two extreme ends of the reactivity curve, the effective dose between the 8th and 14th weeks varies from 0·1 to more than 2 units, whilst at parturition the strips of muscle responded to doses varying from 0·003–0·01 unit. During the earliest stages of gestation too, the phenomenon previously observed in the rabbit's uterus, namely that the reactivity was more marked to vaso-pressin than to oxytocin, was also found on several occasions in the human uterus. Unfortunately no data were obtained before the 8th week. It has, however, previously been shown by Knaus [1929] that the human uterus does not react to pituitrin (and hence neither to oxytocin nor vaso-pressin) during the second part of the menstrual cycle when the corpus luteum is active. It seems a likely inference that the earliest stages of activity of the corpus luteum of pregnancy are also associated with an absence of the reactivity to both posterior lobe hormones. If this be so, then the reactivity of the human uterus during pregnancy can be divided into stages corresponding to those observed in the rabbit, namely:

(1) an absence of reactivity to both oxytocin and vaso-pressin during the earliest stages followed by

(2) a greater reactivity to vaso-pressin than oxytocin and

(3) a gradual increase in the reactivity to oxytocin culminating in labour.

The duration of the various stages are, of course, different in the human and rabbit's uterus as would indeed be expected from the different length of gestation. Another point which deserves emphasis is that the effective dose in the rabbit and human uterus at the various stages shows considerable agreement. For example, the effective dose at parturition for the rabbit uterus varies from 0·001–0·01 unit, whilst for the human uterus the corresponding dose varies from 0·003–0·01 unit. Moreover, in the human as in the rabbit's uterus a few of the strips removed during pregnancy show a much higher reactivity than the average for that stage of gestation (*e.g.* the strips removed from H 36 at the 30th week of pregnancy responded to 0·005 unit of oxytocin). No explanation for the exceptions can at present be offered.

The investigation of the spontaneous rhythmic activity has also given results similar to those previously obtained in the rabbit. For it has been found that the isolated strips do not show any specific type of activity at any particular period of gestation, both high and low activities being observed at the various periods investigated. Moreover, there was no parallelism between the length of the rhythmic contractions and the reactivity to oxytocin, as both a high and low reactivity could be observed in strips showing either little or marked spontaneous activity. It seems therefore likely that the factors which control the rhythmic contractions *in vivo* during the early stages of gestation do not operate *in vitro* and hence that they do not exert a very lasting action on the muscle inasmuch as their action may not persist after removal from the body. Reynolds [1932] has shown that an inhibition of the spontaneous activity of the rabbit's uterus independent of the action of luteal hormones can be observed *in vivo*. It is possible that the factors responsible for the effect described by Reynolds control the uterine activity during gestation, but that their action is not demonstrable on the isolated muscle strips.

If the changes in the reactivity of the uterus take an integral part in the maintenance of pregnancy and initiation of parturition (as has been suggested by Knaus [1930]), then it appears unlikely that the corpus luteum hormones play an important part in controlling them, since it has been shown that bilateral ovariectomy does not necessarily interfere with pregnancy and parturition in the human subject [Ask-Upmark, 1926]. It may be urged that the inhibitory hormone may also be elaborated in the placenta and Mazer and Goldstein [1932] have described one case in which the placenta during pregnancy apparently contained the progestational luteal hormone; the inhibitory reaction was not tested.

Robson and Illingworth [1932], however, investigated the hormone content of four placentæ removed at various stages during gestation and were unable to demonstrate in them any progestational or inhibitory hormone. Similar negative results have been recorded by Clauberg, Thiel and Ziecker [1932]. From the evidence at present available it seems more likely that the changes in reactivity in the human uterus after the early stages of gestation are not primarily dependent on luteal hormones. The previous experiments on the rabbit also suggested that the changes in the uterine reactivity in that animal during the second part of gestation are not dependent on the corpus luteum.

The present investigation also shows that the progressive change in reactivity of the uterine muscle during pregnancy is not specific for oxytocin; for the reactivity to ergotoxin undergoes changes which are at least as definite as those demonstrated for oxytocin, and like that to oxytocin becomes very much higher at parturition than during pregnancy. Furthermore, in a few cases where the reactivity to ergotoxin during pregnancy was high, the muscle also responded to small doses of the oxytocic hormone. These findings suggest that the alterations in the uterine muscle during gestation may involve not merely the reactivity to one particular substance but the reactivity of a definite muscular (or neuro-muscular) excitable system. Such a hypothesis gains support from the findings of Hasama [1931] that the electrical changes in the rabbit's uterus after stimulation of the hypogastric nerve are qualitatively different in the non-pregnant and pregnant animals.

Summary.

The reactivity to oxytocin, vaso-pressin and ergotoxin of strips removed from the human uterus at various stages of pregnancy and at parturition has been quantitatively determined and shows definite progressive alterations. The response to histamine does not show similar variations.

The spontaneous rhythmic activity at the various stages has also been determined, and it is shown that no stage during the period investigated is characterized by any definite type of rhythmic activity. There appears to be no relation between the spontaneous activity of the uterine strips and their reactivity to oxytocin.

The factors controlling uterine reactivity are discussed, and it is suggested that, after the early stages of gestation, the changes in reactivity are not primarily dependent on luteal hormones.

Evidence for the view that the alterations in the uterine muscle during gestation involve the reactivity of a definite muscular (or neuro-muscular) system is advanced.

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CHOLINE AND LIVER FAT IN DIABETIC DOGS.

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In the first completely depancreatized dog which was observed for a prolonged period (70 days) in this department [Banting and Best, 1922], all the symptoms of diabetes were apparently alleviated by insulin. Later, however, several animals were kept in good condition for much longer periods of time. In these animals [Allan, Bowie, Macleod and Robinson, 1924] signs of failure of hepatic function, which autopsy findings suggested were attributable to fatty degeneration of this organ, appeared at varying intervals after pancreatectomy, but the condition did not develop when minced pancreas was added to the lean beef and sucrose diet. In a series of recent publications [Hershey and Soskin, 1931; Best and Hershey, 1932; Best, Hershey and Huntsman, 1932; Best and Huntsman, 1932] results have been presented which support, among others, the following conclusions: (1) that crude lecithine will prevent or alleviate the extensive fatty changes found in the livers of these diabetic dogs, (2) that purified lecithine will prevent the deposition of fat in the livers of normal rats when these animals are ingesting large amounts of fat, (3) that the amount of choline which may be derived from an adequate dose of lecithine will also prevent the fatty changes in the livers of the rats. These results raise numerous questions, one of the most interesting of which is whether choline is, like lecithine, effective in preventing or alleviating the fatty degeneration which may develop in the livers of diabetic dogs. That this question can be answered in the affirmative is the result of an investigation which has been carried out on the ten members of our diabetic colony during the last twelve months.

Methods.

Very little space need be given to the description of methods. Some of these have been described in previous publications. The diabetic dogs were very carefully treated after pancreatectomy and were always in

excellent condition before the experiment was started. Each animal was then placed on a diet of approximately 300 g. lean beef, 100 g. sucrose daily with small amounts of "Bemax," orange juice and cod-liver oil. The insulin was administered 15 min. before each of the two meals and the dose was adjusted to permit a moderate glucosuria. Urinary volume and sugar excretion were determined daily and various other estimations on urine and on blood were made at frequent intervals. Fatty acids of blood were determined by the Stoddard and Drury procedure [1929]. Total fatty acids of the liver were estimated by the Leathes and Raper modification of the Liebermann's saponification method and the iodine numbers were determined by the Rosenmund-Kuhnhenn technique [1923]. As will be discussed later it has been found necessary to remove samples of liver repeatedly from the same animal. Ether anæsthesia was used, and in the earlier experiments a wedge-shaped bit of liver was quickly removed from the most easily available lobe. Bleeding was stopped by oversewing. Later, however, with the help in some experiments of Dr J. Markowitz, a small lobe of the liver was removed after ligation of the pedicle. This latter procedure is much more satisfactory, and even the animals with fatty livers recover very rapidly after the operation. Saline or glucose solution was given intravenously after the operation and the animals were kept quiet for 24 hours by the appropriate use of morphine.

Choline hydrochloride was obtained from the British Drug Houses. The dosage is expressed in terms of the base.

Experimental results and discussion.

The first animal whose history will be outlined is dog "H," which was depancreatized January 12, 1932. The daily sugar excretion and the amount of insulin and choline received are given in Fig. 1. The sugar excretion of these diabetic dogs is usually much higher during the first week or two after pancreatectomy than it later becomes with the same dose of insulin. This is due, in part, to decrease in the carbohydrate reserves. In this animal the glucosuria gradually diminished until the third week after the operation when signs of insulin overdosage were exhibited, and the dose had to be diminished to avoid fatal hypoglycæmia. During the last few days of February a trace of bile appeared in the urine and the animal was unable to retain food. On the assumption that these signs were associated with fatty changes in the liver choline was administered (March 1) and the condition of the dog gradually improved.

There was considerable delay, however, in the appearance of increased sugar excretion. This may have been partly due to inadequate amounts of choline. Later, however, the sugar excretion reached a high level and the animal was apparently in excellent condition. This experiment was then

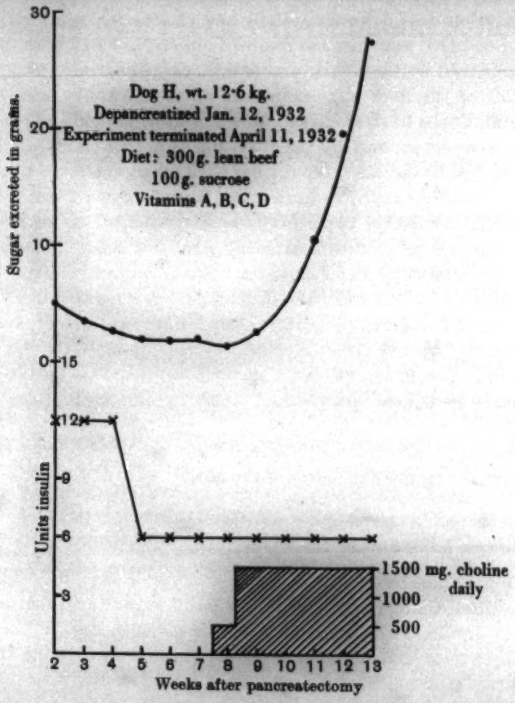

Fig. 1.

terminated and the total fatty acids of the liver were found to be 3·74 p.c. Based on our previous results we believed that this animal had a fatty liver before choline was given and that the accumulation of liver fat interfered with sugar production and possibly with other hepatic functions. If this assumption was correct, the results of this experiment furnish conclusive evidence for the effect of choline on liver fat. In later

experiments analysis of liver samples eliminated the necessity of relying on indirect evidence.

The second case to be considered is that of dog "S," Table I. This animal demonstrated a rather rapid decrease in sugar excretion, and 3 months and 5 days after pancreatectomy the level became so low that 1·5 g. of choline were added to the diet. A week later this amount was increased to 2·25 g., but there was no increase in sugar excretion. The

TABLE I. Dog "S," wt. 16 kg. Depancreatized June 8, 1932.

Weeks after pancreatectomy	Insulin units	Sugar excretion daily (g.)	Fat daily (g.)	Choline daily (g.)	Condition
4	12	56·7	—	—	—
5	12	41·2	—	—	—
6	12	28·9	—	—	—
7	12	29·4	—	—	—
8	12	19·0	—	—	—
9	12	7·8	—	—	—
10	12	3·8	—	—	—
11	12	5·2	—	—	—
12	12	0·7	—	—	—
13	12	0·4	—	1·5	Frequent hypoglycæmic reactions in late evening
14	8	0·5	—	2·25	—
15	5	1·0	10	2·25	—
16	5	0·8	20	2·25	Animal thin but bright and very active
17	5	0·7	20	2·25	—
18	5	1·7	20	2·25	—
19	5	1·0	20	2·25	Bright and active

animal was, however, perfectly well and very active. The insulin dose was decreased from 8 to 5 units daily. Fifteen weeks after pancreatectomy 10 g. and the next week 20 g. beef fat daily were added to the diet. The results of previous experiments indicated that very fatty livers and extensive interference with hepatic function were quickly produced by this amount of fat in animals receiving no lecithine or choline. This animal apparently tolerated the fat very well for 26 days. The experiment was then terminated. A complete autopsy revealed no obvious abnormalities. The value for the liver fatty acids (average of three determinations) was exceptionally low, 1·40 p.c. The iodine number was 115. In this experiment choline appeared to prevent the deposition of liver fat which would otherwise have been produced. But although the liver fat had apparently been reduced even below the normal values, there was no evidence of increased sugar production, a result for which previous experience with lecithine had partly prepared us. It is possible, and even probable when considered in the light of the results on other animals, that this would

have been observed if the experiment had been prolonged, but we can no longer associate, as intimately as previously seemed justified, the elimination of excess liver fat and increased production of sugar.

To illustrate the effects of larger amounts of fat, brief reference may be made to dog "L." This animal followed the usual course for the first month after pancreatectomy with the exception that the sugar excretion remained at a considerably higher level than in other dogs under similar conditions. After approximately 5 weeks fat was added to the diet (20 g. daily). The sugar excretion diminished somewhat and the amount of fat was doubled. On this diet, 300 g. of lean beef, 100 g. of sucrose, 40 g. of fat and 14 units of insulin, the dog became very ill and died. Choline, 1·7 g. a day, was administered for the last 2 days of its life. The fatty acids of the liver were 12 p.c., iodine number 66. If choline had not been given the fatty acid content might have been greater. None of the dogs has been able to tolerate a daily dose of 40 g. of fat without lecithine or choline, and we are convinced that even 20 g. daily cannot be administered for more than a very short time without deleterious results if a source of choline is not also provided. The histories of several other animals support this conclusion and higher fatty acid values have been observed in the livers in some cases—26 p.c. (iodine number 71) in dog "B," for example, whose history is similar to that of dog "L." There is great individual variation in the extent of the fatty changes in the livers of different animals of approximately the same size which have received the same amount of fat. There are no data available which permit us to decide whether or not large doses of choline will enable a diabetic animal to tolerate indefinitely more than 20 g. of added fat per day.

This brings us to a consideration of dog "Sa" whose history provides very convincing evidence for the efficacy of choline. This animal, which is still alive and in good condition, was depancreatized on May 26, 1932. For 4 months the dog was maintained on the usual diet and there was a gradual falling off in sugar excretion. On September 21, 20 g. of fat were added daily to the diet, but it was found necessary to reduce this to 10 g. to avoid vomiting. From October 3, 1·5 g. of choline were given and 2 weeks later (October 18) it was found possible to raise the fat to 20 g. a day without causing gastro-intestinal disturbances. The animal was in very good condition from that date until December 6, when a lobe of liver was removed for determination of fatty acids. The average value was found to be 16·5 p.c. with an iodine number of 66. As soon as the animal recovered from the operation the choline was stopped but the fat was continued. Although the animal made a rapid recovery from the operation, the high

fat diet without choline soon began to produce signs of liver failure and by January 4, the animal was in an extremely poor condition and was hardly able to stand. The condition of the animal at this time was in striking contrast to that displayed 1 month previously. The second specimen of liver was secured on January 4 and was found to contain 20·6 p.c. fatty acid, iodine number 57. Choline was then added to the diet in

TABLE II. Dog "Sa," Wt. 15 kg. Depancreatized, May 17, 1932.

Weeks after pancreatectomy	Insulin units	Sugar excretion daily g.	Fat daily g.	Choline daily g.	Liver fat p.c.	Blood fat mg. per 100 p.c.	Condition
16	16	3·9	—	—	—	284	Excellent
17	16	7·9	20	—	—	256	Fat caused vomiting
18	16	1·7	10	—	—	—	—
19	16	1·1	10	1·5	—	—	—
20	16	0·8	10	1·5	—	—	—
21	16	0·6	15	1·5	—	—	—
22	16	0·3	20	1·5	—	—	—
23	16	0·5	20	1·5	—	—	—
24	16	0·7	20	1·5	—	—	—
25	16	0·8	20	1·5	—	—	—
26	16	1·1	20	1·5	—	—	—
27	16	0·7	20	1·5	—	—	—
28	16	0·5	20	1·5	—	—	—
29	16	7·4	—	1·5	16·5 I.N. 65	—	Rapid recovery from operation
30	16	1·2	20	—	—	—	—
31	16	0·6	20	—	—	95	—
32	16	0·8	20	—	—	136	—
33	16	3·1	—	—	20·6 I.N. 57	158	Vomiting, very weak, condition serious
34	12	0·6	20	2·5	—	—	—
35	12	0·7	20	2·5	—	—	Very weak after operation, saline intravenously
36	12	0·4	20	2·5	—	—	—
37	16	0·6	20	2·5	—	—	Gradual improvement
38	16	0·6	20	2·5	—	—	—
39	16	0·8	20	2·5	—	—	—
40	16	0·5	20	2·5	—	—	—
41	16	0·6	20	2·5	6·7 I.N. 77	373	Condition excellent

slightly larger doses than during the first period of administration and, as our supply of choline was getting low, approximately 1 g. of betaine was also fed daily. The condition of the animal rapidly improved in spite of the 20 g. of fat consumed each day. There was, however, no increase in sugar excretion. On March 5 another lobe of liver was secured and was found to contain 6·7 p.c. fatty acid, iodine number 77. The animal made an uneventful recovery from the fourth operation and appears to be in excellent health. To recapitulate, the history of this dog shows that when the diet, including 20 g. of fat, and insulin dosage were

maintained constant, a small amount of choline kept the liver fat at approximately 16·5 p.c. When choline was withheld the animal became seriously ill and the liver fat rose to 20·6 p.c. When the dose of choline was subsequently increased the fatty acids of the liver fell to 6·7 p.c. and the animal was restored to excellent health.

TABLE III. Dog "J," Wt. 17·5 kg. Depancreatized April 15, 1932.

Days after pancreatectomy	Insulin units	Choline g.	Sugar excretion daily (g.)	Added fat in diet g.	Wt. kg.	Condition
30	8	0	1·2	—	15·3	Good
30–50	8	0	1·2	—	15·4	"
50–70	8	1·27	5·3	—	16·0	"
70–90	8	1·5	4·6	—	15·8	"
90–110	8	1·5	3·2	—	15·1	"
110–130	8	1·5	1·6	—	15·0	"
130–150	8	1·5	4·4	20	14·8	"
150–170	8	1·5	3·9	20	14·8	"
170–190	8	1·5	2·0	20	15·1	"
190–210	8	1·5	4·5	20	15·4	"
210–230	8	1·5	4·8	20	15·5	"
230–250	8	1·5	6·8	20	15·7	"
250–270	8	1·5	20·6	20	15·4	"
270–290	12	1·5	26·2	20	15·4	Excellent
290–310	14	1·5	25·9	20	16·0	"
310–330	14	1·5	20·8	20	15·8	"

Only one other animal's history will be reviewed. In this dog (Table III) choline administration was begun about 7 weeks after pancreatectomy, although no symptoms of hepatic insufficiency were observed. The animal remained in excellent condition. About 11 weeks later 20 g. of beef fat were added daily to the diet. The daily dose of 1·5 g. of choline has apparently been sufficient to protect the animal against this amount of fat, because she has remained in excellent condition on it for 200 days, at the time of writing. When choline was first provided the increase in sugar excretion was very slight. When the fat was also added to the diet there was perhaps a slight increase of sugar output. This would probably not have been observed if choline had not been available. Four months later, the sugar excretion had risen to such a high level that it was considered wise to increase the dose of insulin. The condition of the animal was excellent. The insulin was raised gradually from 8 to 14 units per diem and the glucosuria was still rather high. On the basis of experience with other animals we believe that this dog without choline would succumb rapidly to the effects of accumulation of fat in the liver. The animal has now been under observation for over a year and is still in good condition. It has already shown that a diabetic dog receiving choline can be maintained in excellent condition on a diet

containing much fat for a longer period than it has been possible to keep similar animals on a very low fat diet without choline. The choline content of the lean beef may be of interest in this connection but the amount has not as yet been accurately determined. The total fatty acid fraction of the daily ration of 300 g. is approximately 4·5 g., iodine number 75.

The beneficial effect of the administration of choline to these diabetic animals has been shown to result in a diminished risk of accumulation of fat in the liver, as shown by direct analysis. Up to the present time data obtained by indirect methods do not enable us to form more than a rough idea of the condition of the liver cells in these animals. The estimations of the fat in the blood that we have made are interesting in that they show that there may be little fat in the blood with a very fatty liver, but this subject need not be discussed here because an attempt to correlate the results of blood and liver-fat determinations with those of various liver function tests and with the histological appearance of the tissue in these diabetic dogs is being made.

The nature of the experiments, some of the results of which are reported here, makes it imperative that data on many points not in the direct line of the main investigation should be collected. Much that is new has been learnt about the general care and treatment of diabetic dogs. Pathological changes in kidneys, arteries and eyes have been observed in some animals, but these have not yet received the attention which they merit. A report on the results of a histological study of the livers of dogs "H," "S," "Sa," "B," "L," and others is in the press.

Summary.

The condition in diabetic dogs which is associated with accumulation of liver fat and degeneration of liver cells can be prevented or cured by providing choline in the diet. This conclusion is supported not only by the results of analysis of liver tissue secured from animals which had been restored to health by choline, but samples of liver have been obtained from the same animal during the stage of fatty infiltration and degeneration and later after the amount of liver fat had been reduced by choline. A diabetic dog receiving choline and a considerable amount of fat in its diet has been maintained in excellent condition for a longer period than it was previously found possible to keep these animals, even when they got very little fat, without providing a source of choline. The fatty acid content of the liver may be lowered by choline without an accompanying increase in sugar

excretion, though such an increase may be observed in some animals. These results are briefly discussed, and one direction which further work will take is indicated.

It is a pleasure to express our thanks to our colleagues Miss M. E. Huntsman and Dr E. T. Waters for their very helpful interest in this work. The efficient assistance of the technical staff of the department is gratefully acknowledged.

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REVERSIBLE INEXCITABILITY OF TACTILE ENDINGS IN SKIN INJURY.

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The present work originated in an attempt to understand the phenomenon noticed by Adrian, Cattell and Hoagland [1931], namely, that if the surface layers of frog's skin were scraped away, the tactile responses ceased for a time but returned eventually. The observation was easily confirmed. A piece of frog's dorsal skin with one of its nerves was gently stretched on an ebonite frame about 1½ cm. square in a moist chamber, the nerve being made to lie on two electrodes leading to amplifier and loud speaker, or Matthews' oscillograph and screen. The tactile responses caused by lightly touching various spots of the skin with a pointed glass rod were noticed. The external surface of a portion of the skin was scraped with a sharp knife, until the tactile response there was abolished. After a certain period of time, shorter or longer depending upon whether the preparation was well irrigated or not after scraping, the response would be found to have returned more or less completely.

Three additional points were soon noticed, however, and these supplied the clue to an explanation. The first is that the abolition of the tactile response is not confined to the area actually scraped, but extends some way into the neighbouring regions. Let us imagine the piece of skin under consideration to be divided into three parts, *A*, *B*, *C*, with the nerve entering *B*, the middle portion. In some experiments *B* was scraped. In that case, the responses in the adjacent areas *A* and *C* were generally found to be abolished with that of *B*. This may perhaps be considered natural since the nerve supply to *A* and *C* may come by way of *B*. But in other experiments in which *A* or *C*, instead of *B*, was scraped, the inhibition of responses was also found to extend into *B*, for a greater or smaller distance, sometimes even over the whole area of *B*. The second point is that maximum inhibition often does not occur immediately after scraping, but shortly after. For instance, in one experiment, on testing *B* immediately after it was scraped, it was found still to give feeble re-

sponses, the areas *A* and *C* responding about half as well as before scraping. Five minutes afterwards, however, the whole piece of skin was found to be inexcitable. The third point is that it is very doubtful whether the return of response after abolition by scraping could occur if the preparation was not irrigated or washed after scraping. In several experiments the preparation was not given Ringer after the abolition of response by scraping, but just left in the moist chamber, being tested every 5 min. or so for recovery over a period of 1½–2 hours. There was no recovery. At the end of that time the whole preparation was thoroughly washed with Ringer, and within 10 min. the ability to respond returned.

The above observations limit the field in which an explanation of the phenomenon may be sought. For instance, explanations assigning direct mechanical injury to the endings as the cause of the initial inhibition and their slow reformation as the cause of subsequent recovery are made extremely improbable. Indeed, we are immediately led to the hypothesis that the scraping of skin liberates some toxic substance which causes the inhibition, and whose diffusion away brings the recovery. The correctness of the hypothesis appears to be indicated by the following additional observations. First, in cases where recovery after scraping occurred eventually, it could be made to happen in a very short time, by thorough washing with Ringer. Conversely, whenever the latter procedure failed to bring about recovery within 10 min. or so, no recovery occurred however long one might wait. Secondly, frog's skin when severely scraped, as it was in the experiments, is toxic to sensory endings, though a piece of intact skin is not. This was ascertained by the simple experiment in which an ordinary skin-nerve preparation was set up in the moist chamber, and its excitability tested. A piece of frog's dorsal skin about 1 cm. square was taken and scraped with a sharp knife, in the same way as was done for the study of recovery. It was then applied to the interior surface of the skin-nerve preparation. Within 5 min. the preparation had become inexcitable. Its excitability was now brought back by washing with Ringer. Another piece of skin was taken but this time applied to the preparation without being scraped first. No inhibition was noticeable in half an hour. This same piece of skin was then removed, scraped and applied again to the preparation. The response of the latter again disappeared within 5 min. This was repeated four times with the same results.

We are therefore justified in accepting the hypothesis put forth above as a definite conclusion, namely, that the reversible inhibition of the frog's tactile endings when its skin is scraped is due to the reversible action of some toxic substance liberated by the scraping.

Having arrived at this conclusion, one naturally wonders whether this liberation of toxic substance by scraping is peculiar to the skin, or can be shown in other tissues; and what is the nature of the toxic substance or substances.

A number of tissues were accordingly crushed and tested for their toxicity, if any, by the method already used for the piece of scraped skin, viz. by applying them to the interior surface of a frog's dorsal skin-nerve preparation. The tissues tested include frog's skeletal muscle, lung, liver, tongue, oviduct, laked blood corpuscles, nerve, Achilles tendon, and rat's skin, muscle, and liver. They were all found to be as toxic or more so than scraped frog's skin, that is, they abolished the tactile response in as short or shorter time, and like scraped frog's skin, their effects were all reversible.

So far as frog's skin was concerned one would perhaps suppose that the toxicity was due to some organic compound liberated from the glands. While that might well be the case, the similarity of action of the many diverse tissues which were tested predisposed one to imagine a common factor for them all.

Now it seemed improbable that this common factor could be a complex organic poison. Some physical or ionic factor was therefore suspected. *p*H changes could not be of importance, for muscle poisoned by iodo-acetic acid before being crushed was just as toxic as normal muscle, and Ringer made distinctly red or yellow to phenol red by addition of NaOH or HCl had no apparent injurious effect. Nor could it be osmotic change, for both Ringer diluted with equal volumes of H_2O and Ringer with the concentration of all its constituents doubled only caused continuous discharges of tactile impulses at a diminishing rate for 15 min. or so, but no sign of inhibition in over an hour. Among the ions, excess potassium is well known to have a depressant action, and Dulière and Horton [1929] have shown that it is responsible for the reversible inexcitability of muscle which is not irrigated. Since the potassium concentration in cells is generally much higher than in the body fluids, injured tissues may be expected to liberate potassium in such excess as to cause local paralysis. It was therefore decided to test the idea that the reversible inhibition of tactile responses caused by scraped skin and injured tissues was due to potassium. Firstly it was ascertained that Ringer containing 10 times the normal amount of potassium produced the same reversible inhibition of nerve endings as that under consideration. Consequently the potassium hypothesis may be immediately conceded in cases like muscle, for Horton [1930] has shown that frog's sartorius contains about 320 mg./100 c.c.

of potassium, or about 40 times that in Ringer. Crushed muscle with this concentration of potassium free to diffuse cannot avoid being toxic, in view of the fact that Ringer containing only 10 times the normal concentration of potassium is already so. In the case of other tissues where no data for potassium concentration are available, the doubt may be entertained as to whether there is enough potassium to account for the toxicity observed. As a first test of this point, frog's oviduct, lung and skin, three tissues chosen at random, are completely ashed over Bunsen burners, and the ashes dissolved and neutralized in a weight of H_2O approximately equal to 80 p.c. of the weight of the fresh tissues used for the ashing. Such ash solutions were found to exert the same toxic effect even more rapidly than crushed fresh material. This renders very strong support to the potassium hypothesis, for isotonic NaCl was found to be non-toxic, and a mixture of equal volumes of Ringer and isotonic $CaCl_2$, while toxic, is considerably slower in its action than injured tissues, which furthermore do not contain such high concentration of calcium; it is difficult to see what substance other than potassium could be responsible. Still for final confirmation, rough potassium determinations of the ash solutions were carried out. 1 c.c. each of the ash solutions and a standard solution containing 200 mg. KCl per 100 c.c. were measured in graduated centrifuge tubes and 2 c.c. of 20 p.c. sodium cobaltinitrite reagent were added to each tube with shaking and allowed to stand for 2–3 hours and then centrifuged together. The amounts of precipitates in the tubes were compared. The result was that the ash solution made from oviduct gave roughly about three times as much precipitate as 200 mg. KCl/100 c.c. solution, while that made from skin gave at least as much. A KCl concentration of 200 mg./100 c.c. is considerably more than sufficient to produce the toxic effect whose explanation is being sought.

We may therefore conclude that the reversible abolition of tactile responses produced by scraping frog's skin is due to the liberation of potassium or is at least sufficiently accounted for by it. Whether the glands may also liberate some substance contributing to the poisoning effect or not may be left an open question. Similar statements apply to all other injured tissues examined.

The reversible inhibition of responses after the scraping of skin can be observed with equal ease in mammals. The description given above for frog's skin may be transferred with but slight modification to cat's skin, on which three experiments were made.

NOTE ON THE POSSIBLE RELATION BETWEEN POTASSIUM AND PAIN DUE TO INJURY.

The reversible abolition of tactile responses, however, only represents one side of the effect of skin injury. Scraping the skin, while rendering the tactile impulses inelicitable, causes a persistent discharge of the slow ones. This appearance of slow impulses in scraped frog's skin was observed by Adrian [1931], who obtained various types of slow impulses by applying strong pressure, heat or 2 p.c. acetic acid. There appear to be several kinds of such impulses, each called forth by some specific stimulus. But the slow discharge after scraping the skin possesses a peculiar interest in its long continued persistence, and the search for a persisting stimulus suggests itself. In view of the preceding results one naturally wonders if this persistent stimulus might not be potassium.

Injections of isotonic potassium chloride solution into the skin are known to cause pain [Rhode, 1921; Bommer, 1924][1] and the slow impulses in the frog are probably related to pain rather than to tactile sensation. So far, however, we have found no evidence in favour of the view that potassium causes the discharge of slow impulses. In about a dozen tests on frog's skin and four or five experiments on cat's skin, we failed to obtain any definite evidence of a persistent discharge by laying on the skin pieces of filter paper or cotton wool which had been soaked in KCl solutions of various concentrations up to isotonic. Nor did we succeed any better on introducing potassium intradermally by means of a syringe. Possibly the type of slow impulses appearing in scraped skin are not really pain impulses, and potassium might in fact initiate some very small impulses conveying pain without our being able to record them, or potassium might really have nothing to do with pain directly, the pain sensation on injection of potassium being a secondary effect only, due to, say, potassium contracture of the smooth muscle fibres in subcutaneous tissues.

One more observation, however, deserves mention, that is, the differential sensitivity to the toxic action of potassium of the cutaneous nerve endings giving slow and rapid impulses. Both in frog's and cat's skin moderate pressure gave a discharge of the slower impulses long after the quick ones had disappeared following the application of potassium. Indeed the greatest regularity of slow discharges, due to severe pressure for instance, was obtained by first abolishing the quick impulses by means of potassium. That this differential sensitivity was no accidental thing, owing to the nerve endings responsible for quick impulses being more exposed, seemed to be evidenced by the fact that the earlier abolition of quick impulses occurred whether potassium solution was applied to the exterior or interior surface of a skin preparation.

CONCLUSION.

The chief finding recorded in this paper, namely, the reversible abolition of tactile impulses in skin injury due to potassium liberation, is obviously related to the reversible inexcitability of muscle worked out by Dulière and Horton. The present work, together with theirs, should be a sufficient emphasis upon the part that potassium is likely to play in all experiments involving tissue injury or increased tissue permeability.

[1] Also personal communication of Prof. R. Bárány to whom my thanks are due.

The author takes pleasure in expressing his sincere thanks to Prof. E. D. Adrian, who suggested this investigation, for his advice and interest throughout the work. His thanks are also due to Dr A. Keys for much useful and pleasant discussion.

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612.38:612.59

THE INFLUENCE OF TEMPERATURE ON THE RATE OF EXCRETION OF PHENOLSULPHONEPHTHALEIN IN THE FROG.

By E. M. SCARBOROUGH.

(From the London (R.F.H.) School of Medicine for Women.)

In a previous paper [Scarborough, 1932] reasons were given for considering 15° C. to be the optimum temperature for the investigation of drug effects on the various factors of the intact circulation in the frog. The present series of experiments was undertaken to determine, if possible, the rate of absorption from the lymph spaces at 15° C., and the effect of temperature on the rate of absorption.

Attempts to time the absorption rate of various dyes were made without success, either because of the loss of colour after absorption, or because of the rapid impairment of the heart by the dye. Phenolsulphonephthalein, which is used as a test for the functional activity of the kidneys [Rowntree and Geraghty, 1910], was finally chosen, as it had no apparent effect upon the heart and circulation in the doses used. The time actually measured was the minimum interval between injection into the lymph space and the appearance of phenolsulphonephthalein in the urine, as shown by a purple coloration with sodium hydroxide.

Method.

The experiments were carried out during November and December, 1932, upon male frogs of fairly uniform size. With modifications for collecting the urine, the method used was that previously described [Cullis and Scarborough, 1932], whereby observations may be made on blood-pressure, heart rate and web circulation at a constant temperature. In a preliminary series of experiments the urine was collected from the Wolffian bladder at varying intervals after injection of the dye. This method was unsatisfactory because of the frequent absence of fluid in the bladder, but, when present, the fluid gave a purple reaction characteristic of urine in 15–25 min. after injection at 15° C. This finding may be of interest as evidence in favour of the urinary function of the frog's bladder.

The main series of experiments consisted of three groups of twleve at 10, 15 and 20° C. respectively, and two groups of six at 5 and 25° C. In these experiments the method was modified by the introduction of ureteric cannulæ (Fig. 1), care being taken to avoid bleeding. The ends of the cannulæ projected from the copper-box apparatus, and the urine was withdrawn by means of the pipettes. The thoracico-abdominal cavity was filled with Ringer's solution after the insertion of the cannulæ, as the presence of available fluid was found to assist the establishment and maintenance of the urine flow. The time taken for preparing an experiment and adjusting the frog to the required temperature was about an hour. Observations on the blood-pressure, heart rate and web circulation were recorded at intervals for half an hour. Half a c.c. of a 0·05 p.c. solution of phenolsulphonephthalein was injected into the lymph space of the thigh, and the time elapsing between the injection and the first appearance of the dye in the urine from each ureter was measured. The appearance of a purple colour when a drop of sodium hydroxide solution was added to

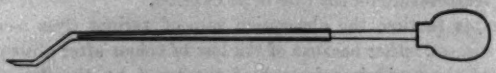

Fig. 1. Ureteric cannula and pipette with rubber bulb attached. (Actual size.)

the urine was taken to signify the presence of the dye. A final circulatory observation was made, the temperature throughout being kept constant.

Control experiments at 15° C. were made in which no circulatory observations were attempted. Ureteric cannulæ were inserted, and after half an hour phenolsulphonephthalein was injected, and the time interval between injection and excretion was measured. In some experiments at 15° C. the cannulæ were withdrawn and emptied completely, in order to estimate the time necessary for the urine to travel from the tip of the cannula to the tip of the pipette.

Results.

Throughout the experiments at 10, 15 and 20° C. the circulation was good; the heart rate and temperature relationship was maintained, the web circulation was not impaired, and there was no excessive fall of blood-pressure. At 5° C. the web circulation was impaired in every experiment, and there was a rise of blood-pressure in five out of six. At 25° C. the circulation was good in three experiments, and in three there was excessive fall of blood-pressure and impaired web circulation.

Fig. 2 shows the time interval between injection of phenolsulphonephthalein and its first appearance in the urine. The average time intervals at 5, 10, 15, 20 and 25° C. were 17·5, 14·5, 11·2, 6·8 and 8 min. respectively, and the shortest recorded times at each temperature were 14, 10, 8, 4 and 4 min. The positive urine test was preceded in all cases by a negative test taken a minute earlier. The first positive test occurred in urine from the right ureter in twenty-two experiments, and from the left in twenty-six. There was no evidence to suggest any difference in urine flow between the two sides. The excretion of phenolsulphonephthalein continued as long as it was possible to maintain the circulation and urine flow. In one experiment at 20° C. the urine gave a positive test 6 hours after injection.

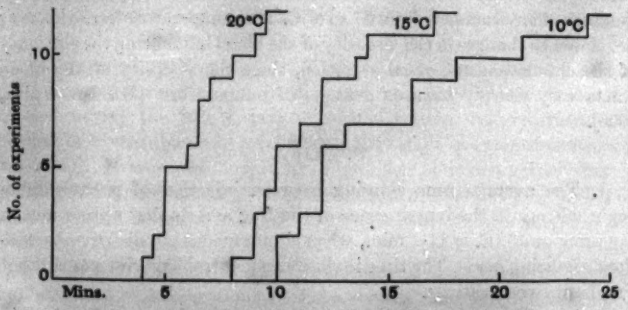

Fig. 2. Time between injection of phenolsulphonephthalein and its first appearance in the urine at different temperatures.

The average time interval of six control experiments at 15° C. was 7·5 min., and the shortest recorded time 5 min. The experiments in which the cannulæ were withdrawn and emptied gave negative tests up to 4 min., positive over 6 min., and varying results for intermediate times.

Discussion.

The average time for the detection of phenolsulphonephthalein in the urine at 15° C. is 11·2 min., under conditions in which simultaneous circulatory observations are made, but at least one minute of this time is taken up in filling the tip of the ureteric cannula. This result is comparable to that of Bieter and Hirschfelder [1924] who record that phenolsulphonephthalein can be seen in the capsular fluid 15 min. after injection

into the submaxillary lymph sac. The time is 50 p.c. longer than in control experiments without circulatory observations, it therefore seems that the method involves some impairment of the circulation. Since, however, the period over which the circulatory conditions can be kept fairly constant is 2 to 3 hours, it would seem reasonable, in further investigations of drug effects, to attribute any changes in circulatory conditions occurring within 10 min. of injection at 15° C. to direct action of the drug.

The time elapsing between injection and onset of excretion varies with the temperature, being slowest at 5° C. and quickest at 20° C. At 25° C. the time is longer than at 20° C. owing probably to impairment of the circulation. Haan and Bakker [1924] find that in winter frogs the velocity of elimination of dye substances varies with changes in the temperature of environment from 17 to 0° C. This temperature variation may be related to changes in the viscosity of the blood influencing the efficiency of the circulation and renal secretion, since the viscosity of the blood reacts very sharply towards changes of temperature [Burton-Opitz, 1906].

Summary.

1. The average time elapsing between injection of phenolsulphonephthalein into the lymph space of the frog and its first appearance in the urine at 15° C. is 11·2 min., when simultaneous circulatory observations are being made. The time is shorter in control experiments without circulatory observations.

2. This time varies with the temperature, being longer at 5 and 10° C. and shorter at 20° C. The shortest time recorded is 4 min. at 20° C.

3. It is inferred that the time necessary for the absorption of a drug lies well within the range of time over which the circulatory conditions can be kept constant.

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THE HISTOLOGICAL CHANGES IN THE BONE RESPONSIBLE FOR THE ACTION OF PARATHYROID HORMONE ON THE CALCIUM METABOLISM OF THE RAT.

By L. I. PUGSLEY and HANS SELYE.

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It was reported by Pugsley [1932] that the calcium excretion in rats receiving daily injections of parathyroid hormone[1] was markedly increased during the first 4 days of administration, but returned to a normal or even subnormal level within 8–10 days in spite of continued injections. It was also found by a number of investigators that if parathyroid hormone was given to animals or man over a long period of time it lost its power to mobilize calcium, and in the later stages of the treatment the serum calcium might actually be reduced by the hormone instead of being increased. The explanation of these observations was unknown and it has been assumed that a certain "immunity" is developed against this hormone after a certain period of treatment.

Selye [1932*a*, *b*] found that the explanation of the resistance acquired in the later stages of experimental hyperparathyroidism in rats was that the hormone eventually led, not to the formation of osteoclasts (that is, cells which destroy bone) but to the proliferation of osteoblasts which build new bone tissue.

The following experiments were planned to determine whether these changes in the histological reaction of the bone appeared at the same time as the changes observed in the calcium excretions and serum calcium—that is, whether the osteoclastic reaction (osteitis fibrosa) changed into an osteoblastic one (marble-bone formation) at the same time as the increased calcium excretion and hypercalcæmia returned to normal levels. We have been interested in such a comparative study of the bone histology

[1] The authors are indebted to Eli Lilly and Company, Indianapolis, U.S.A., for the parathormone used in these experiments.

and calcium metabolism because the demonstration of an exact coincidence in the changes produced would give strong support to the theory that the immunity is merely the result of a change in the reaction of the osteogenic tissue to the hormone.

Methods.

Rats of Wistar strain, 110–115 days of age, and weighing 175–225 g., were used in the experiments. The diet and methods for determining calcium in the urine and blood were those described by Pugsley [1932]. Each rat received 20 units of parathyroid hormone daily during the experimental period, the beginning of which is marked by an arrow in Fig. 1. Histological studies of the bones were made on the group of rats used in following the excretion of calcium in the urine. One animal was sacrificed each day during the experimental period for these studies. The serum-calcium values were obtained from a different group of rats kept under identical conditions and also receiving 20 units of the hormone daily. The results of the serum-calcium determinations are shown in Table I, and the average values represented graphically in Fig. 2.

The calcium excretion in the fæces was not followed in these experiments because previous work by Pugsley [1932] showed that it did not run such a consistent course as the calcium excretion in the urine, nor did it display such a striking response to the injections.

Discussion.

The histological examination of the bones of these animals showed, in accordance with our previous findings, that the first reaction of the bone to parathyroid hormone is the formation of numerous osteoclasts. This reaction is quite pronounced 2 days after the administration of the hormone. As the treatment is contined the spindle-cell proliferation becomes more pronounced, until on the fourth day of administration the picture obtained is as seen in Fig. 3. The formation of numerous spindle-shaped osteoblasts in the bone marrow is a constant feature of osteitis fibrosa, and the present series of experiments shows that their appearance does not interfere with the increased calcium excretion or the rise in serum calcium. It was found that during the period where both osteoclasts and osteoblasts are increased in number the calcium excretion remains at abnormally high levels. However, the disappearance of the osteoclasts, which was observed on the ninth to twelfth day of treatment, coincided exactly with the return of the calcium excretion and the serum calcium to

TABLE I. Effect of daily administration of parathyroid hormone upon the serum calcium of rats.

Days of administration	No. of determinations	Serum calcium		
		Highest	Lowest	Average
Control	40	10·7	9·6	10·3
1	3	11·2	10·8	11·0
2	3	13·9	13·2	13·5
3	3	14·6	13·4	13·9
4	4	14·4	13·9	14·2
6	3	15·1	12·6	13·8
10	4	11·8	11·3	11·6
12	4	10·9	10·4	10·6
17	5	10·4	10·1	10·2

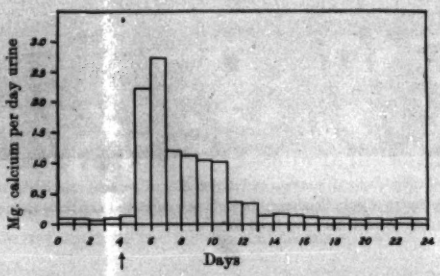

Fig. 1. The effect of administration of 20 units of parathyroid hormone per day upon the excretion of calcium in the urine of adult rats.

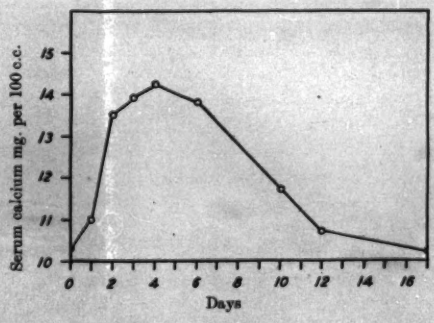

Fig. 2. The effect of administration of 20 units of parathyroid hormone daily upon the serum calcium of adult rats.

Fig. 3. Histological picture of bone on the fourth day of parathyroid hormone administration. Thick layers of spindle-shaped osteoblasts surround the bone tissue; at the same time numerous osteoclasts have been formed.

Fig. 4. Histological picture of bone at the eleventh day of parathyroid administration. The osteoclasts have disappeared and only osteoblasts can be seen.

normal levels. On the fourteenth day the bone picture was that shown in Fig. 4. Here we find the bone trabeculæ surrounded by many layers of more or less spindle-shaped osteoblasts and practically no osteoclasts are present. If the treatment is discontinued at this stage the bone structure returns to normal; if it is continued the osteoblasts increase in number and lead to the formation of huge amounts of bone tissue, so that the typical picture of "marble-bone" results [Selye, 1932*a*, *b*].

SUMMARY.

A comparative study of the changes produced in the histological structure of the long bones and the calcium metabolism of the rat has been made. It was found that the return of the previously increased calcium excretion and increased serum calcium to normal levels in the later stages of experimental hyperparathyroidism coincides exactly with the disappearance of the osteoclasts in the bone marrow.

The authors wish to express their sincere appreciation of the kind interest and help of Prof. J. B. Collip and Dr D. L. Thomson during the course of this investigation. We also wish to thank Prof. F. E. Lloyd for assistance in preparing the photomicrographs and Mr T. McKeown for assistance in preparing the histological sections.

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612.627:612.63.031.1

CAPACITY OF THE UTERUS OF THE RABBIT TO RESPOND TO PROLONGED LUTEAL ACTIVITY.

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Introduction. Attempts to prolong pseudopregnancy in the rabbit by inducing successive ovulations have led to different conclusions as to the capacity of the uterus to respond to continued luteal activity. Parkes [1929] by starting injections of anterior pituitary extract towards the end of normal pseudopregnancy maintained the development of the uterus and increased the growth of the mammary glands to that of pregnancy. Courrier and Kehl [1930], however, failed to obtain significant prolongation of uterine activity in the pseudopregnant animal or in a sterile horn during pregnancy. The above authors made daily subcutaneous injections of anterior pituitary extracts during the period of experiment. This technique has the obvious disadvantage that relays of corpora lutea and luteinized follicles are produced indiscriminately and that no systematic continuity of luteal action is effected. In the light of recent work, the appropriate technique is to cause successive ovulations by single intravenous injections [Bellerby, 1929] at intervals of about 10 days. Experiments carried out on these lines have given results similar to those obtained by Parkes.

Methods and materials. A series of eight isolated rabbits was used. Each animal was injected intravenously with 5 c.c. of fresh urine of pregnancy, and the ovaries were examined 24 hours later by aseptic laparotomy to determine if ovulation had occurred. This procedure was repeated every 10 days and the animals killed at periods varying from 3 to 5 weeks after commencement of the experiment. Material for histological examination was fixed in Bouin's fluid and stained with hæmatoxylin and eosin. Sections were prepared from two regions of each cornu.

Fig. 1. Uterus of rabbit 12 showing typical pseudopregnant proliferation 28 days after first injection. × 10.

Fig. 2. Uterus of rabbit 14 showing partial pseudopregnant proliferation 38 days after first injection. × 10.

Results and conclusions. The experiments are summarized in Table I.

TABLE I.

No. of exp.	No. of injections	No. of ovulations	Killed—days after first injection	Proliferation of uterus
3	2	2	20	+ +
6	3	3	26	+ + +
7	3	3	28	+ + +
8	3	3	28	+ + +
11	3	3	28	+ +
12	3	3	28	+ + + +
5	4	3	37	+
13	4	4	35	+ +
14	4	4	35	+ + +

In comparing the degree of proliferation of the uterus the full pseudopregnant state is represented as "++++" and the œstrous condition as "0" [Allen, 1930]. From an examination of the table it will be seen that uterine activity has been maintained in all experiments, although the reaction in five is very slight. This animal, however, failed to ovulate after the last injection and will therefore have been devoid of active luteal tissue at the time of autopsy. The remaining animals showed varying degrees of pseudopregnant proliferation (Figs. 1 and 2). Examination of the uteri shows that the uterine response is less strong 35 days after the first injection than at 28 days, and sensitivity may therefore be falling off after 5 weeks. Under the conditions of this experiment, however, it obviously exists for at least 5 weeks.

SUMMARY.

Pseudopregnant proliferation of the uterus of the rabbit may be greatly prolonged as a result of the formation of new corpora lutea in the ovary.

My sincere thanks are due to Dr A. S. Parkes for suggestions and help in this work.

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THE PHYSIOLOGICAL SIGNIFICANCE OF WEBER'S LAW AND COLOUR CONTRAST IN VISION.

By J. S. HALDANE.

It was originally shown by Bouguer [1760] that over wide limits the degree of visibility of a shadow thrown by a local obstruction to a source of light depends, not on the intensity of the illumination produced by the light, but on the ratio between the illumination of the surface on which the full illumination falls, and the illumination of the shadow. The visibility of the shadow thus depends on this "geometrical" ratio, and is not proportional arithmetically to the difference between the illumination of the shadow and that of the surface illuminated by the light. He also found that, as has often been confirmed, the smallest difference which can be appreciated steadily in this ratio is about 1·5 p.c. In his recent book on *Photometry* Walsh [1926] puts this figure at 1·6 p.c., as a result of experiments at the National Physical Laboratory with the improved form of the Lummer-Brodhun photometer.

Weber [1846] showed that a similar conclusion applies to other kinds of sensory stimuli. It thus came to be known as Weber's Law of Sensory Stimuli, and to be regarded as a fundamental law covering response to differences in sensory stimuli. It was later taken by Fechner and other psychologists as a fundamental psychophysical generalization covering the supposed relation of body to mind. I propose in this paper to discuss, in the light of further experiments, the physiological significance of Weber's Law as applied to visual sensation, including its extension to colour sensation.

It is known that under certain conditions—for instance with extremely feeble illuminations—the law ceases to hold; but I wish first to point out usual conditions where, even with strong illuminations, it does not hold at all, and the visibility of a shadow depends simply on the arithmetical difference between the illumination on a shaded and on an illuminated surface.

The experiments described in this paper have been to a large extent carried out with the help of the simple form of Rumford or Lambert shadow photometer which I recently designed for testing rapidly the

candle-powers of miners' lamps [1928, 1933]. With this photometer the shadow of a standard light is thrown by an upright bar about ¼ in. wide on an upright white screen, and a shadow of the lamp to be tested is thrown side by side with it on the same screen, the shadows being thrown at a very acute angle to one another, and being looked at from above and directly in front. The lamp is then moved with one hand along an ordinary photometer scale (with graduations numbered proportionally to the squares of their distances from the shadow) till the shadows are equal in depth. If the standard light is the usual one (for miners' lamps) of one candle-power, the reading on the scale, with the standard light in its ordinary fixed position, gives directly the candle-power of the lamp, and would be 1·00 on an average if the lamp were exactly of one candle-power. We are comparing the illuminations of two white surfaces, each of which is illuminated by one of the lamps; and by varying the candle-power of the standard, or its distance from the screen, we can easily verify the fact that in accordance with Weber's Law the minimum steadily visible difference in illumination is, within wide limits, a fraction of about 1·5 p.c. of the illumination given by either lamp, as was originally pointed out by Bouguer.

But now let us throw a very feeble shadow, from a distant or very feeble light, across the white surface and top of the strong shadow from one near light. It will be seen that this feeble shadow is no more easily visible on the strong dark shadow than on the strongly illuminated white surface. Thus Weber's Law does not hold at all in this case. A difference which, when measured with the photometer, can be shown to be about 1·5 p.c., is just visible on the strongly illuminated white surface; but on the strong shadow, where the percentage difference in illumination caused by the feeble shadow is immensely greater, the feeble shadow is not more visible, provided that the strong shadow is not very wide. As we widen this shadow, however, the weak shadow becomes relatively more and more visible on the strong shadow.

We can easily show that the diminished visibility of the weak shadow on the stronger one is due to the surrounding illumination of the white screen by the near lamp. If we cut off this illumination, the feeble shadow at once stands out as a very strong one, in accordance with Weber's Law. In ordinary daylight the general field of vision is strongly illuminated, and we can now see that as regards differences of illumination within details of this field, Weber's Law does not hold at all. It is easy to verify this by experimenting with a feeble shadow, like that on the photometer screen. Under ordinary circumstances, therefore, it is not

geometrical but arithmetical differences in illumination that we appreciate. The latter are what we are accustomed to call the "objective" differences. The conditions under which Weber's Law can be verified are quite special; and when we realize this we can see the need for a much wider physiological generalization than is embodied in Weber's Law.

If we balance the shadows of two lamps on the photometer, we can easily, without direct interference of any kind with the shadows, upset the balance seriously by placing a piece of black paper on one side of the screen. The effect is to make the nearest shadow relatively brighter; and a difference of as much as 20 p.c. in the relative brightness of the two shadows may be produced, as measured with the photometer, if the black surface reaches as far as the shadow nearest to it. This experiment shows how necessary it is, in using the photometer, that the two shadows should be well in the centre of a uniform field. It has also been familiar to me for long that in using a colorimetric method (for instance with a Gowers-Haldane hæmoglobinometer), the tube on one side commonly appears darker than that on the other, owing to differences in illumination between the two side backgrounds. Hence it is necessary to transpose the tubes at each observation; and colorimeters, however elaborate, which do not permit of this may give rise to much error.

Although Weber's Law does not hold under ordinary conditions, a fact which does hold is that within wide limits of general illumination in the physical sense, the visibility of shadows, and consequently of the illuminated objects which we see, remains practically the same. When the illumination becomes very feeble, this generalization no longer holds. If, however, we observe more closely, we find that in darkness so complete that we cannot see any objects at all, the field of vision is nevertheless illuminated by what is known as the "intrinsic" illumination or light. This intrinsic illumination can be seen, when the eye has adapted itself to the darkness, to consist of minute and evanescent areas of light and darkness, producing the general impression of grey, with considerable apparent brightness, which, if we judge by the contrast between dark and bright within it, seems to be about the same as that of the average field of vision in daylight. We can now realize that in very feeble illumination external objects disappear because the illumination from them has become a physiologically inappreciable fraction of the intrinsic illumination, and that in less feeble illumination objects are indistinct because the intrinsic illumination has partly covered up the differences in their own illumination.

In reality we are in the presence of a very fundamental physiological fact with regard to vision. This is that though from a physical standpoint the external light acting on the eye varies throughout enormous limits, from zero to that of full daylight, which is about 100,000 times greater than the illumination by which on an average an English coal-miner works at present, the physiological illumination of the field of vision remains, so far as can be judged from the contrast of brightness with darkness, about the same. Brightness of bright areas, and darkness of dark areas, are relative always to a general field of visual activity which, apart from the temporary effects of sudden change in illumination, is persistent. What appears as light or dark is thus an expression of contrast within this field. When we pass rapidly from what, in the physical sense, is bright illumination to dim illumination, physiological after-effects of the bright illumination remain for some time, adding to the intrinsic illumination; also, as is well known, fading in intensity and often producing changes in colour. Hence time elapses before vision of surrounding objects becomes "adapted" to dim light—in other words before such vision becomes possible as the unreinforced intrinsic illumination permits.

Before further discussion I shall refer to experiments on colour-vision. It is well known that though, from a physical standpoint, the quality of daylight, light from incandescent filaments or mantles, and from various kinds of flame, varies very considerably, yet what we call a white surface seems, or may seem, about equally white in light from any of these sources. The phenomena of coloured shadows, the existence of which has been known for centuries, appear, however, to reveal in a most striking manner the differences between the different qualities of light; and in using a photometer, when the relative brightnesses of different kinds of light are being compared, the differences in colour of the shadows are very prominent. In reality coloured shadows reveal a great deal more than colour differences, as will now be shown.

If we allow a shadow produced by the light of a candle to fall on the photometer screen, and beside it a shadow produced from daylight coming through a narrow chink left open in a shutter, or from an electric lamp with blue glass giving about the same quality of light as daylight (a "daylight" lamp), the shadow thrown by the candle will, as is well known, appear blue, and that thrown by the daylight orange-yellow, while the rest of the screen appears white. One shadow is illuminated by the daylight alone, and the other by the candle alone. If the two shadows are not equally bright, one has a dark, and the other a light

tint; but if we now adjust the two lights (by varying the distance of the candle or in some other way) so that the shadows are of about equal brightness, the one shadow will have a very surprising deep blue colour, and the other a deep orange-yellow colour, though in spite of the difference in colour we can, with a little practice, estimate the relative brightnesses quite well. The colours are equally evident even if the shadows are widely separated from one another, and they are quite clearly due to simultaneous contrast.

We can vary this experiment in various ways. For instance, we can substitute for daylight the light from an electric light with a red glass. The shadow illuminated by the candle alone will appear as a deep green, and the other shadow as a deep red. If we use a green electric lamp the shadow illuminated by the candle alone will appear as a deep red, and the other as a deep green. With an orange electric lamp, the shadow illuminated by the candle will be dull blue, and the other shadow yellow-brown. With a lemon-yellow electric lamp the shadow illuminated by the candle becomes dull violet. For the candle light we can substitute the almost pure spectral light from a sodium flame, but the result is much the same. We can make the surface illuminated by the candle or sodium flame or daylight any tint we please, from red to blue or violet, by varying the quality of the other light. To anyone seeing these experiments for the first time they are most striking and surprising. With the help of a suitable lantern screen the whole of them can be shown to a large audience. The earliest, and still in many ways the best, description of coloured shadows produced experimentally is that of Rumford [1794], and he writes with enthusiasm about them.

It is now evident that the colour of an illuminated object does not depend simply on the physical quality of the light reflected from it; and indeed every artist paints as if he knew this. Coloured shadows and similar contrast effects produce a grossly exaggerated or completely distorted impression of the "physical" differences in different kinds of light. Coloured shadows are somewhat of a bugbear in photometry, not so much because it requires some practice to distinguish differences in intensity of illumination from differences in colour, as because with different colours different intensities are not quite the same for different individuals. Very striking quantitative data illustrating this fact are given in a recent paper by Dudding, Winch and Cooper [1931].

By covering the screen with non-reflecting black paper, except at the place occupied by the shadows, we can cut off any influence of the surrounding illuminated and apparently white surface. This does not

alter the colours seen except that they both appear brighter, and the shadows now appear as illuminated surfaces. The colour effect, therefore, depends in this case on the contrast between the two shadows. If, however, we separate the two shadows widely, and then block out one of them from the field of vision, the colour of the visible shadow is unaltered, so that its colour is due to contrast between the shadow and the surface, illuminated by both lights, around it. When the brightness of the shadows is balanced the surface has double the illumination of either shadow; but one half of this is due to the illumination on the other shadow, and to the remaining half must be attributed the fact that the contrast effect of the apparently white surface on a shadow is the same as that of the other shadow alone.

If we cut off the light entirely from one of the lamps, the shadow which is left appears simply black, while, if the light illuminates a sufficiently large white surface, at some distance, this surface may appear almost white, though the lamp itself, and any white surface close to it, appears strongly coloured. If, now, we make the shadows very broad, we can see that the contrast effect is most marked close to the edges of each shadow, and diminishes with distance from them. The contrast effect is thus a partly localized one.

We obtain a similar result if, with the help of a black diaphragm, we illuminate one half of a large white screen with ordinary electric light, and the other with blue or red electric light. When the intensities of illumination are about the same the part illuminated by the ordinary electric lamp appears as yellow or green, particularly near where the two illuminations meet, and the other half as blue or red; but with much stronger illumination by ordinary electric light the corresponding part of the screen becomes of a lighter and the other half of a darker tint.

We can vary the experiments in another manner. We can cut a hole in the photometer screen, and then arrange a source of light falling on it so that the light passes obliquely through the hole, and does not fall on any visible part of a white surface placed behind. This surface is, however, so arranged that it is illuminated by a chink of daylight, or a concealed "daylight" lamp, arrangements being also made so that the intensity of the latter illumination can be varied by altering its angle of incidence on the illuminated surface or in some other way. By this means we can vary the brightness of the surface seen through the hole without altering the brightness of the light falling on the screen. What can now be seen is very striking. Let us suppose that the screen itself is illuminated by an ordinary electric lamp, and thus appears white.

The surface seen through the hole will now appear blue. But the more the illumination of this latter surface is reduced, the deeper does the blue become, while the more its illumination is increased, the lighter does the blue become. If, now, when the surface seen through the hole in the screen appears deep blue, we turn off the electric light illuminating the screen, the surface seen through the hole becomes rapidly pure white. The blue is of course the complementary colour to the yellowish electric light, but all the time the surface of the screen appears pure white. If we throw coloured light on the surface beyond, its colour is twisted round towards blue.

Instead of illuminating the screen with ordinary electric light we can use light of any other quality. The screen itself will appear more or less white, while the surface seen through the hole will have the complementary colour to that of the light; and the less brightly the surface seen through the hole is illuminated, the deeper is its complementary colour. Only when the surface beyond begins to be more brightly illuminated than the screen does the complementary colour begin to appear round the hole.

We can also vary the experiments in a negative manner by cutting out the influence of the illumination surrounding what we are looking at. This can be done by looking with one eye through a narrow tube, blackened inside, and at the same time taking care that no light enters the eye at the near end of the tube. A tube about a centimetre in internal diameter and 30 cm. long is convenient, and can easily be made by rolling up a sheet of dull black paper, preferably lined inside with black velvet, and gumming it in position. Blackened tubes were first used by Rumford [1794] for experiments on coloured shadows.

If, now, we look through the tube at the orange-red shadow thrown by daylight or a daylight lamp on the photometer screen, we see that it rapidly fades to a much less intense form of yellow, similar to that given with the tube when the candle alone illuminates the screen. It is the same with all the varied tints of coloured shadows produced when we substitute for daylight various coloured lamps. We have eliminated the contrast effect between the tint of the candle illumination and that of the screen. We get a similar result with the shadow cast by the candle. The heightened colour of this shadow fades into the white or much less intense colour seen through the tube when the screen is only illuminated by the daylight or coloured lamp.

It is also the same when we look at the variegated colours seen through the hole in the screen in the experiments described above when

the surface behind is illuminated by a daylight lamp. They all fade into white or something very near it when the hole is looked at through the blackened tube, though the colours appear again if the tube is so held that part of the screen comes into view. In actual fact, the "daylight" lamps supplied to me give, in spite of their blue glass, a distinctly blue shadow with ordinary daylight; and a white surface illuminated by the lamp looks, through the tube, distinctly yellow.

We can now see a physiological explanation of a phenomenon which has always been more or less of a puzzle—namely the blueness of the sky. If we take the blackened tube and look up at a piece of clear blue sky, the colour soon fades into pure white, just as in the experiment in which the hole in the screen is looked at through the tube. But the blue reappears again if we allow a white cloud to come into the field of vision, just as when we allow part of the screen to come into the field of vision in the experiment with the hole in the screen. The light coming from the clear blue sky has much less of the violet end of the spectrum cut off than light coming through clouds or obliquely from the sun, since the violet end is more readily scattered by particles in the air. Physically speaking, therefore, the light coming through clouds, or obliquely from the sun, is yellower than that from the clear sky. Even a clear sky looks blue, since all surrounding objects illuminated by the sun have their colours twisted round, like the colour of the clouds, towards white. Through a blackened tube this effect is undone, so that white becomes yellow, blue changes towards white, green towards yellow, red towards orange, and purple towards red.

It is well known that when the sun is low, and particularly if it is shining through rather misty air, it looks red, and throws a corresponding green shadow on a white surface illuminated from the sky above. When the sun is higher and looks yellow, the shadow is blue or violet; whereas with the sun high up the shadow becomes black. There is no difficulty in explaining the coloured shadows as simultaneous contrast effects, but when we turn to the blue colour of the sky it seems at first difficult to explain this as a contrast effect, since brightly lit clouds look quite white, or colourless. When, however, we examine these clouds, or a white surface lit by them, on an overcast day, with the blackened tube, we can see that they gradually turn yellow, which is not the case with the clear overhead sky.

But if the clouds are "really" yellow, which they appear to be when the disturbing effects of the peripheral field of vision are eliminated in the blackened tube experiment, why do they not look yellow? And why,

in general, do white surfaces look white when they are illuminated by yellow electric light, or still more yellow candles? Why, also, do they look more or less white when they are even illuminated by red, or green, or blue lamps? When any of these surfaces are examined with the blackened tube they appear in what might be regarded as their "real" yellow, red, green, or blue colours. This very pertinent question will be discussed presently.

A very striking phenomenon, which can often be observed in tropical countries with a clear sky as the sun is just disappearing at sunset, or appearing at sunrise, is known as the "green ray." The light coming directly from the disappearing or appearing upper edge of the sun appears for two or three seconds of a brilliant green colour. One of Jules Verne's novels takes its title from this appearance. In a recent paper Lord Rayleigh [1930] pointed out that the light in the violet half of the sun's light must be more refracted by the atmosphere than the light in the red half, and hence must be visible longer at sunset, and earlier at sunrise. He also devised an experimental arrangement which enabled this effect to be studied at leisure. The colour seen was, however, blue, not green. It now seems evident, in view of the experiments described above, that the cause of the actual sunset or sunrise coloured ray being vividly green, is the simultaneous contrast effect due to the light coming from round the sun being red.

When, with the ordinary coloured shadow experiment we keep the relative intensities of illumination of the shadows the same, we see their tints equally distinctly over a very large variation of illumination, and if we make both shadows very faint by removing both sources of light to a distance, or in any other way, the difference in colour remains still visible so long as the two shadows are separately visible. It is thus relative, and not absolute differences in illumination that determine the visibility of the colours in the shadows. We can, therefore, to judge from this experiment, extend Weber's Law to colour appreciation when we regard each colour and its complementary colour as similar in its physiological behaviour to brightness and darkness.

On the other hand, this extension of Weber's Law appears as if it were quite unjustifiable when we consider the fact that when colours are produced locally by pigments, each colour appears the same regardless of what other colour is adjacent to it. We can, for instance, place red and yellow patches together without the yellow being turned green, whereas in the coloured shadow experiment yellow light becomes green.

We are at once reminded of the fact, already discussed in connection

with uncoloured shadows, that although, within wide limits, the visibility of a shadow depends, not on the intensity of general illumination, but on the contrast between the general illumination of the neighbouring surface and that of the shadow, yet when a shadow is produced locally without sensible alteration of the general illumination, the visibility of this shadow depends directly on the (arithmetical) difference between its illumination and that of the neighbouring surface. Similarly a difference in visible colour produced locally by altering the quality of the light illuminating a large surrounding or neighbouring surface depends, not on differences which can be arithmetically expressed between the two lights, but on the contrast between the colour actually given by one of the qualities of light and its complementary colour given by the other; yet when the visible colour is produced locally without sensible alteration of the general illumination, the colour depends directly (arithmetically) on the difference in quality between the illumination given by the surface and that given by a white surface under the same general illumination, or, failing illumination from without, by the intrinsic illumination. It appears, therefore, that we must interpret the presence or absence of colour contrast in the same way as presence or absence of contrast with bright and dark illumination.

In accordance with this statement, coloured shadows possess tints which are complementary to one another in so far as they are tinted at all. But patches of colours produced by pigments in the same general illumination have a tint which is independent of the co-presence of other patches of colour. In darkness, moreover, a luminous patch, such as that produced by a coloured lamp, has what we are accustomed to call its "natural" colour.

The physical difference between a coloured surface illuminated by a white light and a white surface illuminated by a coloured light is that in the former case that part of the white light which by itself would produce a colour complementary to that of the coloured surface is largely absorbed, and the rest largely reflected, thus producing the colour seen. In the latter case nearly all the coloured light illuminating the white surface is reflected; so that the surface illuminated exclusively by each coloured light might be expected to appear of the same tint as the respective coloured light. In actual fact, however, the colour of neither surface may correspond to the light which is illuminating it, and the colours which appear may be complementary to one another.

The difference as regards presence or absence of contrast effect between a coloured surface produced locally by a pigment and one

produced by coloured light in a coloured shadow experiment depends on the facts already discussed. As is well known, however, to painters as well as physiologists, contrast effects may be visible in shadows thrown by ordinary daylight or other light of one quality, next to a coloured surface. In this case the contrast effect is not produced by two different kinds of light transmitted to neighbouring surfaces, but by the two different kinds of light reflected from two neighbouring surfaces. Contrast effects of this kind are, however, much less readily produced, and less striking than those produced by two different kinds of light transmitted to neighbouring white surfaces.

By throwing a shadow of suitable depth on a white background by the side of a pigmented surface we can obtain a distinct though rather feeble contrast effect, the colour of the pigment near the junction being deepened, and the complementary colour appearing in the nearest part of the shadow. Necessarily, however, the complementary colour appears dark as it is in shadow; and the deepening in the colour of the pigment is correspondingly slight. It is easy to produce this localized contrast effect by putting some tissue paper over a pigmented surface and a black surface next to it. A better method, as is well known, is to use a rotating disc for producing the darkened surface. In all these experiments it is necessary to distinguish carefully the simultaneous contrast which is characteristic of them from the successive contrast which is apt to be confused with it.

The explanation of the localized contrast effect at the edge of a shadow which is neither too light nor too deep is that though a local pigmented surface has no sensible effect on the general standard of colour appreciation in the field of vision, it has a distinct localized effect close to the edge of the coloured surface, thus producing a contrast effect which is noticeable when the intensities of illumination on the two contrasted surfaces are about equal, just as the far more prominent colour contrast of coloured shadows is most striking under the same conditions.

Without using lights of different colours we can produce the coloured shadow effect with a sufficiently large pigmented surface round a white surface, if we shut off other surrounding illumination, so that only the pigmented surface and sufficient of the white surface are visible. We can make the intensities of illumination on the two surfaces equal by inclining the white surface, so that it appears darker in accordance with Lambert's "cosine law." The contrast effect extends over the whole of the white surface, as in a coloured shadow experiment; but the result is not

so striking, since there is, for comparison, no surface which appears white.

The reason why the deep colours produced by pigments disappear in growing darkness before the coloured objects themselves disappear is the fact that the intensity of illumination of the colours in the pigmented objects is so much less than in white or light-coloured objects. Towards night all deeply pigmented objects become black.

Just as we can disturb the balance of depth of illumination on the photometer screen by introducing a black surface on one side, so also we can disturb balance of tint by introducing a coloured surface on one side. Thus if we produce on the photometer screen two shadows of the same tint, we can alter this tint by introducing a coloured surface on one side. As I showed many years ago, we can by tintometric titration with carmine solution determine with great accuracy the percentage saturation of hæmoglobin with carbon monoxide. Examples of this are given in a paper by Douglas and myself [1912]. But in comparing the tints of the blood solutions in the two test-tubes, differences in the colour of the background behind the sides of the two test-tubes have so distinct an effect that it is always necessary to transpose the positions of the tubes at each observation, just as with an ordinary colorimetric determination. A further point which comes out clearly in this method is that with different persons the amount of carmine which has to be added to produce equality of tint differs quite markedly, though the final result as regards percentage saturation is exactly the same. Similarly in photometric work, with a difference in colour between the two lights compared, there are, as already mentioned, appreciable differences between practised observers as regards the candle-power of the lamp. For some persons, for instance, intensity of light from a flame lamp as compared with that from an electric standard is definitely greater than for others.

Discussion.

We can unify or explain the whole of the phenomena relating to Weber's Law in its relation to vision, and to coloured shadows and simultaneous contrast generally, if we assume that a normal constancy tends to be maintained in the field of visual activity, in such a manner that when one part appears brighter or coloured, the rest, and particularly what is immediately adjoining, tends to appear darker or of the complementary colour to a corresponding extent. Let us consider in detail how this theory covers the facts.

We may first take the facts which Weber's Law embodies. The

theory at once covers the fact that over an enormous range of general illumination in the physical sense the visibility of brightness and darkness remains practically the same, and that over a similar range of general illumination the visibility of complementary colour difference remains practically the same, although, as Purkinje discovered, the range is not quite the same for all colours (unless, as we have seen, they are complementary).

The theory also covers the fact that of whatever quality in a physical sense the general illumination may be, unpigmented objects, such as white paper, tend to appear as white. Except when we first come into it, or look at objects through a blackened tube, we are not even aware that ordinary electric light is yellow, and candle or other flame-light still yellower. Nor have we ordinarily any suspicion of the yellowness of the light on an overcast day. But even when a room is illuminated by a deep yellow, red, green, or blue electric lamp, the colour of white paper looks nearly white, and its deeply coloured illumination in a physical sense is only realized on looking at it through a blackened tube.

In the next place the theory covers the fact that in the details of what is visible as bright or dark in the field of vision, or as coloured, Weber's Law does not hold at all. Both degrees of bright and dark, and degrees of colour are seen in accordance with that we are accustomed to regard as the physical difference between them, and not as ratios between bright and dark, or between colour and complementary colour. This is at once intelligible when we consider that any mere local variation in the field of vision has, unless it is of great relative magnitude, almost no influence in disturbing the general balance of brightness, or colour in the visual field. In ordinary daylight it is the overwhelming influence of the general illumination which standardizes our vision of the details of brightness and colour. It is only when the sun's rays are falling very obliquely, and much less intensely, with a clear sky above, that we are aware of their yellowness or redness by contrast with the light coming down more or less vertically from the sky; but the fact that the clear sky, or light reflected from the sea, is blue, reveals the physical character of the yellow light from clouds and sun. The light from the blue sky is feeble as compared with the yellow light coming directly, but obliquely, from the sun, or through the medium of bright clouds. Hence it is the sky that looks blue, and not the sunlight or clouds that look yellow, just as in the experiment with a hole in the photometer screen.

With a relatively intense light in one part of the field of vision, the rest of the field is of course darkened, and an intense coloured light, if it

is localized by some means, turns the rest of the field of vision towards its complementary colour. This also becomes intelligible on the theory. The fact, on the other hand, that colour contrast effects due to ordinary differences in colour in small parts of what is visible and illuminated by the same light are localized at the junctions of the parts, and only visible clearly when the brightnesses of the two parts are about equal, is equally intelligible.

Finally, the fact that when we look at things through a sufficiently narrow blackened tube we see things in what for physics is their objective colour, becomes quite intelligible. White in the physical sense is such a mixture of light of different wave-lengths as gives the sensation of white. Our sensations correspond with those produced by this mixture when we practically shut out disturbing contrast effects by admitting to the eye only a very small and even part of such light shining from without in the ordinary field of vision. We get this sensation when we apply the tube to the pure "blue sky", or to a white surface illuminated exclusively by it, but by no means by looking through the tube at anything which may appear to us white without use of the tube.

In surrounding darkness we obtain a similar "objective" perception of the colours of small parts of the field of vision. We notice at once, for instance, that electric light is yellow, and we see coloured lamps in their "objective" colours, though we can easily change these colours into quite different ones by altering the surrounding illumination. If, for instance, we paste a piece of translucent paper over a hole in the photometer screen, and illuminate this paper by coloured light from behind the screen, we at once change the colour of the paper to white or to various other colours than that of the lamp behind, by changing the colour of a lamp illuminating the front of the screen.

When there is no light falling on the eyes, and any after-effects of previous illumination have ceased, intrinsic illumination remains, in accordance with the theory, and is simply a colourless grey, which in no way affects the colour of any localized illumination from outside. Hence by using a sufficiently narrow blackened tube, we can see what can be regarded as the "objective" colours of what we see, though these "objective" colours are simply the colours seen under certain physiological conditions, so that we have made no real separation between objective and subjective. If the tube is insufficiently narrow, this method of course defeats its own object, since a large illuminated surface will tend to appear white, whatever its "objective" colour may be. In using the blackened tube we must also be careful to avoid the influence of

successive contrast on the intrinsic illumination. If, for instance, after looking in the ordinary way at a coloured shadow, or at blue sky, we use a blackened tube in looking at it, we at first see the same colour, and a little time elapses before this colour changes into the "objective" colour. It is the same if we look at an apparently white surface which is illuminated by coloured artificial light or daylight from an overcast sky, or at a pigmented object illuminated in the same manner. Through the blackened tube they all take on only gradually the "objective" colour, the reason being that the after-effect on the intrinsic illumination takes some time to disappear, with the result that the colour, as well as the brightness, of what is seen through the tube is at first greatly affected by simultaneous contrast. In his discussion of coloured shadows Helmholtz [1867] was entirely deceived by this fact, which led him to interpret the blue colour of a candle shadow in presence of daylight as a psychological effect. This had the unfortunate effect of leading physiologists to neglect the very striking phenomena of coloured shadows, although Hering [1887] pointed out later that the blue colour seen through a blackened tube disappears rapidly. He attributed it, though not in a quite correct manner, to successive contrast.

This leads me to a discussion of successive contrast, which has hitherto been only incidentally referred to. On the theory which has been developed above, simultaneous contrast is an expression of the fundamental tendency in visual activity to maintenance of a normal general balance. Thus the physiological phenomena of brightness and darkness, and of colour and complementary colour, are part of the adaptation which is occurring everywhere in visual phenomena. But adaptation in any direction, once established, has a tendency to persist. Hence the intrinsic illumination, on looking through a blackened tube, is at first markedly affected by the previous illumination, with the result that the simultaneous contrast effect previously present remains for a short time. In darkness the after-images of previous visual excitations are positive, in accordance with this interpretation, although they may afterwards become negative, as instanced in the striking experiments described by Creed [1931].

We can now regard, not only the phenomena of simultaneous contrast, as embodied in Weber's Law applied to the visual phenomena of both brightness and colour, but also the phenomena of absent simultaneous contrast effects in ordinary perceptions of brightness and colour, as manifestations of the maintenance of constancy in activity within the visual spatial field. Successive contrast, on the other hand, can be

regarded as an indication of the tendency to constancy in the temporal field of visual activity. The word "contrast" is thus only a convenient expression without fundamental significance, and Weber's Law is only a partial representation of physiological facts, not valid at all under quite ordinary conditions.

It is evident that the theory now adopted is inconsistent with Young's three-colour theory of vision, as also adopted by Helmholtz and Maxwell. The phenomena of colour contrast, as observed in connection with coloured shadows and similar effects of both simultaneous and successive contrast, are left unexplained by this theory. We are thus driven towards Hering's conception of two pairs of primary colour sensations, besides a pair of sensations representing brightness and darkness, and always present in conjunction with colour sensation. What is simply white and black represents a balanced combination of the two primary pairs of colour sensations.

The theory stated above makes it possible to relate the maintenance of constancy in visual activity to various other respects in which an organism maintains constancy in its active relations with physiological environment—for instance the fundamental fact that throughout all the variations in intake and output of material and energy what is characteristic in structure and activities tends to be maintained in an adult organism. The manner in which, as Claude Bernard first pointed out, the composition and other conditions of the blood are kept constant, constitutes a special instance of this. A further instance, exemplified in much of my own work in physiology, is the manner in which the pressure of CO_2 in the body, together with its hydrogen-ion pressure and oxygen pressure, tend to remain steady. In other words, the actual relations of environment to a normal living organism are such that what is characteristic of its life is maintained, though from the standpoint of mere physical science this cannot be expressed, since physical science is an abstract branch of knowledge which takes no account of what is fundamental in the phenomena of life, and thus, since we are living organisms, enters into all our perceived experience.

To realize the fundamental features in the physiology of vision, including colour vision, we must approach the subject as physiologists, and not merely as physicists or chemists, just as in the case of other physiological activities; and as I have tried to point out in more than one recent book, we get nowhere except into what seem to me the fogs of either vitalism or attempted mechanistic biology, if we treat an organism and its environment as separately existing things. What appears

light or dark, red or green, blue or yellow, depends, not on conditions which we can separately specify in either the environment or organism, but on relations throughout which we can trace the active maintenance of what is constant in visual activity. The field of vision is a field of co-ordinated activity, and not of mere receptivity.

It is evident that in the conclusions reached in the present paper, use has been made of biological, and not mere physical or chemical conceptions. The fact that in the sensory relations between organism and environment we can, as physiologists, discern one aspect of the maintenance of life as such, is part of biology, and not of abstract physical science.

It is well known that Goethe [1808] attacked ineffectively the teaching of Newton with regard to colour, and particularly the first proposition in Newton's *Opticks*, according to which "lights which differ in colour differ also in degrees of refrangibility." In the course of his voluminous writings on Colour, Goethe drew attention to the already well-known phenomena of coloured shadows, but unfortunately did not study them thoroughly enough. We can see from the present paper that criticism of Newton's statement is unanswerable. His statement is only true under definite physiological conditions, and lights of precisely the same refrangibility may have very different colours. But similar criticism can be directed generally against abstract physical reasoning which, following on the lines laid down by Galilei, disregards not only what is characteristic of biological experience, but also the fact that all elements in our experience have a definitely biological or psychological basis. The practical utility, for certain limited purposes, of making this abstraction is indubitable; but in biology we are, whether we are clearly aware of it or not, employing conceptions which can penetrate much more deeply.

Summary.

1. Weber's Law, as applied to vision, only holds under exceptional conditions: we are ordinarily aware of arithmetical, and not geometrical, differences in either intensity or tint of illumination.

2. Coloured shadows and similar contrast phenomena do not represent what we are accustomed to call the natural tints of either of the two surfaces concerned, but exaggerated or distorted differences in these tints.

3. The phenomena represented in Weber's Law, in ordinary vision of differences in illumination and tint, and in colour contrast, are summed

up in the physiological conclusion that the field of visual activity, regarded as a whole, tends to exhibit constancy with respect to the prevalence within it of brightness and darkness, and of pairs of complementary colours, the latter being regarded as together equivalent to a degree of whiteness.

4. Young's three-colour theory of colour vision does not cover the phenomena of colour contrast.

For help in the experiments described in this paper I am greatly indebted to Dr R. H. Makgill.

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THE EFFECT OF ŒSTRIN ON THE REACTIVITY AND SPONTANEOUS ACTIVITY OF THE RABBIT'S UTERUS.

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During pregnancy the uterus of the rabbit and of the human subject undergo specific variations in reactivity to certain substances and especially to the oxytocic factor of the posterior pituitary lobe [Robson, 1933 *a*, *b*]. These variations are, to some extent, dependent on the activity of the corpus luteum acting probably through a definite hormone [Robson and Illingworth, 1931; Robson, 1932 *a*; de Fremery, Luchs and Tausk, 1932]. It appears likely, however, that the changes which occur after the earliest stages of gestation are independent of the luteal activity, and the present investigation was performed to determine the effect of the ovarian hormone, œstrin, on the reactivity and activity of the uterine muscle.

Methods.

The experiments were performed on mature female rabbits weighing about 2 kg. With three exceptions the animals were ovariectomized on the day previous to the beginning of œstrin injections. In a number of cases a horn of the uterus was removed at the time of ovariectomy; part of it was examined histologically and the remainder used to determine the reactivity and activity of the muscle. The animals were again operated on after various periods of œstrin injections and the existing state of the uterus determined. Some of these animals were kept under observation for a further period and the condition of the uterus following cessation of œstrin administration determined.

In other cases no uterine tissue was removed at ovariectomy and the state of the uterus was determined only after œstrin injections and at subsequent periods.

In three further cases (Ra 351, 353, 357) the animals were operated on in the usual way and the condition of the uterus and ovaries noted, but

no tissue was removed. Œstrin injections were started as before, on the day following the operation, and the remainder of the experiment was performed as in the ovariectomized animals.

The uterine strips were suspended in oxygenated Ringer-Locke solution in 100 c.c. containers; the temperature (37° C.) and the oxygen supply were maintained constant. Records were taken on smoked drums. The degree of spontaneous rhythmic activity exhibited *in vitro* was determined in all cases, the strips of muscle being of similar length to that used in previous investigations. In the description the minus sign is used to represent slight or no spontaneous activity, while an increasing number of plus signs are used to denote various degrees of rhythmic contractions. The data are strictly comparable with those described for the pregnant animal [Robson, 1933 *a*].

The following preparations of œstrin were used in the experiments:

(1) An extract from pregnancy urine prepared as follows. Concentrated pregnancy urine was acidified with 10 p.c. H_2SO_4 and extracted with ether continuously for 12 hours. The ether extract was extracted with sodium carbonate solution (which was discarded) and then with NaOH. The latter solution was acidified and extracted with ether; the substance left on evaporating the ether was dissolved in maize oil (= α 4 and α 5. I am indebted to Mr R. E. Illingworth for the supply of these preparations). Assay was by four injections spread over 36 hours.

(2) Progynon kindly supplied by Messrs Schering-Kahlbaum.

(3) A crystalline preparation of œstrin supplied by the British Drug Houses. This was prepared according to the method of Marrian and Hazelwood [1932]. The crystalline substance was dissolved in olive oil and assayed by four injections spread over 36 hours; a potency of 1000–2000 M.U./mg. was obtained, but the former figure has been used in the data given in this paper.

The preparation of the posterior pituitary lobe "pitocin," kindly supplied by Parke, Davis and Co., was used in this investigation.

Results.

In estimating the reactivity of the uterine muscle to certain drugs an attempt was made in all cases to determine the minimal amount necessary to cause a motor effect. In the majority of cases this included a rise in the base line, but in some experiments small doses chiefly caused an increase in the height of the rhythmic contractions, and occasionally an increase in the rate of rhythmic contractions was the main effect. The

TABLE I. To show the effect of œstrin injections on the reactivity to oxytocin and spontaneous activity of the isolated uterus. Animals were ovariectomized except Ra 351, 353, and 357. Animal Ra 380 received progynon up to the 7th day and α 5 subsequently.

(S.A. = spontaneous activity.)

Animal (Ra)	Preparation used	Reactivity before ovariectomy (unit)	S.A. before ovariectomy	Reactivity (unit)	S.A.	Dose of œstrin (M.U.)	Time after ovariectomy (days)	Reactivity (unit)	S.A.	Additional dose of œstrin (M.U.)	Time after ovariectomy (days)	Reactivity (unit)	S.A.	Additional dose of œstrin (M.U.)	Time after ovariectomy (days)
351	α 4	0·1	+ + +	0·1	+ + + +	60	7	0·004	+ + +	160	14	0·02	+ + +	Nil	31
357	α 4	0·1	+ + +	0·004	+ + + +	70	8	0·01	+ + +	350	14	0·02	+ + +	Nil	31
380	Progynon	>0·6	+ + +	0·1	+	420	7								
—	α 5														
384	Œstrin	>1·0		0·02	+	450	11	0·005	+ + +	350	15	0·3	+ + +	Nil	24
385	,,	0·6		0·006	+ + + +	1900	11					0·01	+ + +	Nil	21
386	,,	0·02		0·01	+ + + +	2700	13					0·01	+ + +	Nil	18
387	,,	>0·3		0·005	+ + + +	3200	13					0·03	+ + +	Nil	18
353	α 4			0·01	+	90	10	0·05	+ + +	50	14			Nil	14
365	α 5			0·01	+	230	7	0·002	+ + +	340	14	0·08	+	Nil	33
374	Progynon			0·02	+ + +	600	7					0·3	+ + +	Nil	24
376	α 5			0·02	+	700	8	0·005	+ + +	Nil	18	0·1	+ + +	Nil	24
377	α 5			0·05	+ + +	70	8	0·01	+ + +	400	14	0·3	+	Nil	15
378	Progynon			0·05	+	310	7					0·02		Nil	14
379	α 5			0·02	+ + +	600	7					0·1	+ +	Nil	32
381	α 5			0·05	+ + +	140	8	0·02	+ + +	500	16				
382	Œstrin			0·3	+ + +	390	9	0·005	+ + +	310	16	0·1	+ + +	Nil	27
383	,,			0·02	+ + +	260	7	0·01	+ +	240	14	0·1	+ + +	Nil	27

A

B

C

Fig. 1. To illustrate the progressive effect of œstrin injections on the uterus (Ra 384).

A. Section of uterus before œstrin administration. Area = 466. Reactivity > 1·0 unit. × 15.

B. Section of uterus after 10 days of œstrin administration. Area = 1296. Reactivity 0·02 unit. × 15.

C. Section of uterus after 14 days of œstrin administration. Area = 1800. Reactivity 0·005 unit. × 15.

reactivity to oxytocin of the uterus before ovariectomy was, six out of seven animals investigated, low (see Table I) or very low; in one case (Ra 386) it was quite marked, 0·02 unit of pitocin (to 100 c.c. of solution) being sufficient to bring about a motor effect. After injection periods varying from 6 to 12 days during which doses ranging from 60 to 3200 M.U. of œstrin (in various forms) were administered, the reactivity to oxytocin was increased in every case but one (Ra 351). This animal received the smallest amount, namely 10 M.U. daily for 6 days. The increase in reactivity showed marked variations in the different animals and the degree of decrease in the minimal effective dose ranged from zero (Ra 351) to more than 200 times (Ra 384), and was quite marked in the majority of experiments. In one case (Ra 386) in which the initial reactivity was comparatively high, the œstrin injections did not greatly increase the reactivity to oxytocin. That the increase in reactivity can also be obtained in the presence of the ovaries is definitely shown by animal Ra 357 in which the injection of œstrin decreased the minimal effective dose of oxytocin from 0·1 to 0·004 unit.

In those experiments in which uterine tissue was not removed at the first operation the results subsequently obtained confirm the findings of the first series of experiments. For the average reactivity after œstrin injection was much higher than that shown by the first series of animals before any œstrin injections (Table I) and the minimal effective dose of oxytocin varied from 0·002 to 0·05 unit.

The data also definitely show that both time and dosage play an important part in the development of the reactivity to oxytocin, though marked individual variations among the animals were observed.

Dosage.

It appears likely that a certain minimal dosage of œstrin is necessary to obtain an effect on the uterine reactivity. Thus in Ra 351 six daily doses of 10 M.U. had no appreciable influence on the reactivity to oxytocin; that this may be an ineffective dose is also shown by animal Ra 353 in which the reactivity of the uterus actually decreased during a period when 10 M.U. of œstrin were being injected daily (10th to 14th days of the experiment). That this same dose may, on the other hand, be effective is shown by the results obtained in animal Ra 357 in which the reactivity increased by 25 times within 8 days. The relation of dosage to action on the uterine muscle is presented in Table II. When the reactivity previous to the injections is comparatively high even large doses of œstrin will not markedly decrease the minimal effective dose of oxytocin; thus the

reactivity in animal Ra 386 was only doubled as the result of the injection of 2700 M.U. of œstrin, the initial minimal dose of oxytocin being 0·02 unit.

TABLE II. Showing the relation between the period over which œstrin is injected, the dosage used and the increase in the uterine reactivity to oxytocin.

Animal Ra	Dose M.U.	Duration days	Reactivity before unit	Reactivity after unit	Increase in reactivity times
351	60	6	0·1	0·1	1
380	420	6	>0·6	0·1	> 6
357	70	7	0·1	0·004	25
384	450	10	>1·0	0·02	> 50
385	1900	10	0·6	0·006	100
386	2700	12	0·02	0·01	2
387	3200	12	>0·3	0·005	> 60
357	420	13	0·1	0·004	25
380	770	13	>0·6	0·01	> 60
384	800	14	>1·0	0·005	>200

Time.

Even with comparatively large doses of œstrin, however, the injections may have to be distributed over a fairly long period in order to obtain a high reactivity to oxytocin in the uterine muscle. Thus in animal Ra 380 the minimal effective dose of oxytocin after the injection of 420 M.U. of œstrin during 6 days was fairly high (0·1 unit), but an additional period of 7 days (during which 350 M.U. of the hormone were injected) further reduced the minimal effective dose by 10 times. In another experiment (Ra 382) 0·3 unit of oxytocin was necessary to cause contraction of the uterus after the injection of 390 M.U. of œstrin during 8 days, but the reactivity was decreased by 60 times after a further period of 7 days during which 310 M.U. of œstrin were injected.

After the cessation of œstrin injections the reactivity of the uterine muscle decreases, though the rate and extent of the decrease show marked variations in the different animals. The relation between time and decrease in reactivity are represented in Table III. It will be seen that in 10 cases out of 11 an increase in the minimal effective dose of oxytocin was noted. The exception is provided by animal Ra 357 in which, 7 days after cessation of œstrin injections, the reactivity to oxytocin was unchanged (0·02 unit). Animal Ra 376 shows a somewhat temporary exception though it received 50 M.U. of œstrin on the day after the first removal of uterine tissue; 9 days later the reactivity to oxytocin had actually increased by 4 times, but 25 days after the first uterine strip had been excised the minimal effective dose of oxytocin had actually increased by 15 times. The results do not suggest that there is a direct temporal

TABLE III. To show the fall in the uterine reactivity to oxytocin at various periods after cessation of œstrin injections. The value in column 4 is obtained by dividing column 3 by column 2.

Animal Ra	Reactivity before unit	Reactivity after unit	Decrease in reactivity times	Interval after cessation of injections days
387	0·005	0·01	2	5
379	0·02	0·02	1	7
374	0·02	0·08	4	7
386	0·01	0·03	3	8
378	0·05	>0·3	> 6	8
377	0·01	0·1	10	10
385	0·006	0·3	50	13
383	0·01	0·1	10	13
381	0·02	0·1	5	16
380	0·01	0·02	2	17
376	0·02	0·3	15	25

relation between the time after cessation of œstrin injections and the fall in the reactivity to oxytocin, as comparatively small decreases were observed both at the earlier (*e.g.* Ra 387, 379, 376, 386) and later periods (*e.g.* Ra 380, 381). It must also be emphasized that even after a fairly long time had elapsed after the cessation of injections a comparatively high reactivity may be observed (*e.g.* Ra 330 in which, 17 days after the last œstrin injection, the minimal effective dose of oxytocin was still 0·02 unit).

Spontaneous activity.

The results obtained in those animals in which uterine strips were removed at the beginning of the experiments definitely show that injections of œstrin can increase the *in vitro* spontaneous rhythmic activity of the uterine muscle; this increase was observed in all animals investigated (Table I—Ra 351, 357, 380, 384, 385, 386, 387). Moreover, the increase in spontaneous activity becomes, as a rule, more marked as the period of injection is prolonged. At the same time, when the relation between spontaneous activity and reactivity is examined in those animals injected with œstrin (Table IV), there appears to be no connection between these values. It would thus seem that, although œstrin increases both the spontaneous rhythmic activity and the reactivity to oxytocin, the magnitudes of the increases in these values vary independently of one another.

After cessation of œstrin injections the spontaneous activity decreased in a majority of animals observed. Within the time limits investigated the fall in the rhythmic activity seems to be a slower process than the fall in reactivity to oxytocin; moreover, there appears to be little relation between the rate of decrease of these two values.

TABLE IV. To show the relation between the reactivity to oxytocin and the spontaneous activity in the uterus of animals injected with œstrin.

Animal Ra	Reactivity unit	Spontaneous activity
382	0·3	+ +
351	0·1	+ + + +
380	0·1	+
377	0·05	+ + +
353	0·05	+ + +
378	0·05	− +
381	0·05	+ − + +
353	0·03	+ +
376	0·02	+ − + +
374	0·02	− +
384	0·02	± +
379	0·02	+ + + +
381	0·02	+
383	0·02	+ +
365	0·01	+
353	0·01	+ − + +
386	0·01	+ +
380	0·01	+ +
377	0·01	+ + + +
383	0·01	+
385	0·006	+ +
384	0·005	+ +
382	0·005	+ + +
387	0·005	+ +
357	0·004	− + + +
357	0·004	+ − + + + +
365	0·002	+ +

Alterations in size.

As is well known, œstrin produces a marked increase in the length and diameter of the uterus in addition to its actions on the reactivity and spontaneous activity of the uterine muscle; there also occurs proliferation of the endometrial glands which, however, remain straight and do not assume the appearance characteristic of the period of activity of the corpus luteum. An attempt was made to obtain a rough quantitative estimate of the effect of œstrin on the growth of the uterus by measuring the diameters of sections of pieces removed at various periods; in all cases diameters at right angles to one another were measured and multiplied by one another, the number obtained giving an index of the size of the uterus. It is fully realized that the results so obtained depend, to some extent, on the part of the organ from which the piece has been removed, on the state of contraction of the uterus at the time of removal, etc. At the same time the data are nevertheless significant in so far as large differences are concerned. They show that œstrin injections increase the size of the uterus and that the extent of this increase becomes more marked as the period of injections is lengthened (Table V), these effects

TABLE V. To show the relation of size of uterus to reactivity to oxytocin in animals before and after injection of œstrin and after cessation of hormone administration. (For details of injections see Table I.)

Animal No.	Area of uterus	Reactivity unit	Time after ovariectomy days	Area of uterus	Reactivity unit	Time after ovariectomy days	Area of uterus	Reactivity unit	Time after ovariectomy days
384	466	>1·0	0	1296	0·02	11	1800	0·005	15
385	670	0·6	0	>2400	0·006	11	—	—	—
386	784	0·02	0	1600	0·01	13	—	—	—
387	999	>0·3	0	2912	0·005	7	—	—	—
379	3300	0·02	7	2500	0·02	14	—	—	—
380	2597	0·1	7	2308	0·01	14	—	—	—
381	2548	0·05	8	2400	0·02	16	—	—	—
382	1440	0·3	9	2544	0·005	16	—	—	—
383	1400	0·02	7	>2230	0·01	14	1560	0·1	27

being therefore similar to those obtained for the reactivity and spontaneous activity under similar conditions. In the injected animals, however, there appears to be no direct relation between the size of the uterus, its reactivity to oxytocin and the spontaneous activity exhibited by the isolated strips; and these results suggest that the extent of these increases in different animals varies widely and independently. After cessation of the injections the size of the uterus, like the reactivity and spontaneous activity, decreases.

DISCUSSION.

It has been shown that œstrin exerts three well-marked and concurrent actions on the uterus, namely (1) growth which involves both the endometrium and the muscle, (2) increase in the reactivity of the muscle to oxytocin *in vitro*, and (3) increase in the spontaneous rhythmic activity exhibited by the muscle *in vitro*. All these effects can be observed in the uterus of one and the same animal and are, moreover, progressive in character in so far as they become more pronounced as the period of treatment with œstrin is prolonged. It must be emphasized that these effects were obtained with various preparations of the hormone (including crystalline samples), though there are no data as to the relative activities of the various forms of œstrin.

The absence of any apparent relation in the injected animals between the reactivity to oxytocin, spontaneous rhythmic activity and size of the organ appears at first sight rather puzzling, in view of the fact that these three values are determined by the action of the same hormone. The same absence of any direct relation between the reactivity to oxytocin and the spontaneous activity was observed in the uterus of the rabbit and the

human subject during pregnancy [Robson, 1933 *a*, *b*]; here again the data showed that these values apparently varied independently. If all these effects are actually due to the direct action of the hormone on the uterus, then the reactivity of the organ for these various actions must be subject to wide variations. It would also seem to follow that the reactive systems affected by œstrin and responsible for reactivity to oxytocin, spontaneous activity and growth respectively cannot be identical.

With regard to the action of œstrin on the reactivity to oxytocin two factors appear to be of importance. In the first place a certain minimal dose of the hormone is necessary for the production of an increase in the reactivity; and secondly the action of the hormone must be distributed over a certain period and the reactivity may only increase gradually. This is the case even with fairly large doses as, for example, in Ra 382, where, after the injection of 390 M.U. over 8 days, the uterus only reacted to 0·3 unit of oxytocin while the additional injection of 310 M.U. over 7 days decreased the minimal effective dose of oxytocin to 0·005 unit. These results would suggest that the addition of œstrin *in vitro* cannot cause an increase in the reactivity to oxytocin (if it is assumed that the increases above described are actually due to direct action of the hormone on the uterus) owing to the necessarily limited period of action, and the recent results of Marrian and Newton [1932] show that such indeed is the case and that in very large doses œstrin *in vitro* actually decreases the reactivity of the uterine muscle to oxytocin.

The actual reactivity to oxytocin exhibited after the injection of œstrin is significant: in 12 out of 17 animals investigated the ultimate minimal effective dose of the posterior lobe hormone varied from 0·002 to 0·01 unit, while in four of the remaining five animals in which the reactivity was less marked the œstrin injections had been spread over a short period of 6 days, and it appears quite likely that the reactivity in these animals would have been increased if the injections had been continued for a longer period. Now the reactivity of the rabbit's uterus at or shortly after parturition varies from 0·01 to 0·0001 unit of oxytocin [Robson, 1933 *a*], figures which are very similar to those obtained after the injection of œstrin and which are higher than those observed under any other conditions. The similarity between these findings suggests that the œstrous hormone may be an important factor in the development of the uterine reactivity during the later stages of pregnancy and in the production of the high reactivity to oxytocin which characterizes parturition. Such a supposition is supported by the observation of Phillip [1929] that œstrin can be demonstrated in the blood of the rabbit during

the later stages of gestation. Also it is known that the action of œstrin in increasing the uterine reactivity cannot occur as long as the inhibitory hormone of the corpus luteum is being produced in any appreciable quantity, for œstrin cannot overcome the inhibitory effect of the luteal hormone [Robson and Illingworth, 1931; Illingworth and Robson, 1932], and furthermore the presence of œstrin is actually essential for the effect of the inhibitory luteal hormone itself upon the uterine muscle [Robson, 1932 *b*]. As the rabbit's uterus does not contract up to the 20th day of gestation with large doses of oxytocin (the exact time varies in different animals [Robson, 1933 *a*]), while vasopressin affects the muscle after the 16th day of gestation, it appears likely that the action of the inhibitory hormone ceases sometime between these two dates (*i.e.* between the 16th and 20th days), that the subsequent increase in the uterine reactivity to oxytocin is brought about by œstrin and that no appreciable quantities of inhibitory luteal hormone are then being produced.

In the human uterus the sequence of events appears to be similar. It is definitely established that the corpus luteum is not essential for the maintenance of gestation after the earliest stages [Ask-Upmark, 1926]. Moreover, the reactivity of the uterine muscle to vasopressin is already present at the 8th week of gestation although oxytocin does not, at that stage, exert any effect on the muscle [Robson, 1933 *b*]. These findings suggest that the action of the inhibitory luteal hormone ceases very early in pregnancy and that, as in the rabbit, the duration of its action may not be much longer than that of pseudo-pregnancy (if pseudo-pregnancy in the human subject be regarded as corresponding in length to the active period of the corpus luteum of menstruation). Subsequently the reactivity of the human uterus to oxytocin returns and gradually increases, reaching a maximum at parturition, and it seems quite possible that œstrin may play an important part in determining this series of events. It has indeed been established that the concentration of the hormone in the blood and its excretion in the urine gradually increases during pregnancy, reaching a maximum at parturition [Siebke, 1929; Zondek, 1930]. Moreover, the output of œstrin in the pregnant human subject is not interrupted by the removal of both ovaries at a very early stage of gestation [Waldstein, 1929]. These facts support the view that the reactivity changes in the human uterus during pregnancy are dependent on the action of œstrin. There are no data for the time relations of the action of œstrin upon the reactivity in the human subject, nor for the doses necessary for this action, but it appears at least possible (in view of the results obtained in

the rabbit) that the gradually increasing reactivity to oxytocin characteristic of pregnancy is, to some extent, dependent upon the continued action of the gradually increasing concentrations of œstrin present in the blood. The results also suggest that in the clinical use of œstrin, not only should large doses of the hormone be used, but the treatment should be continued over a fairly long period if an action on the uterine muscle is the aim in view.

The fall in the reactivity to oxytocin found in these experiments after the cessation of œstrin injections is interesting, as a similar phenomenon is observed in the uterus of the rabbit after parturition. In the latter case, indeed, the decline is very rapid and the minimal effective dose of oxytocin is always very greatly increased within 5 days of parturition, while after cessation of œstrin injections the reactivity to oxytocin decreases much more slowly. It seems possible that this difference may be due to the fact that in the injected animals the œstrin is not immediately excreted and that indeed some considerable time may elapse before the hormone is completely eliminated and its action on the uterus ceases. The fact that in one case (Ra 376) in which 50 M.U. of œstrin were injected on the day after the first operation which followed a 7-day period of hormone treatment, the reactivity actually increased after the cessation of œstrin injection and only decreased at a still later stage, supports the view that the action of œstrin on the uterus is continued for some time after the administration of the hormone has ceased. Even after cessation of hormonic action, however, the reactivity to oxytocin probably decreases slowly, and in the case above described (Ra 376) the uterus still contracted with 0·3 unit of oxytocin 24 days after the last injection of œstrin. A response to 1 unit of pituitrin has similarly been observed previously in an untreated animal 22 days after bilateral ovariectomy.

Summary.

The injection of œstrin into ovariectomized and non-ovariectomized mature female rabbits causes (1) an increase in the reactivity of the uterus to oxytocin *in vitro*, (2) an increase in the spontaneous rhythmic activity exhibited *in vitro*, and (3) an increase in the size of the organ. There appears to be no correlation between the degree of these three effects.

The relation of dosage and of the period of injection and the effects of œstrin have been investigated. The progressive nature of the action of œstrin is stressed.

The state of the uterus after œstrin injections is compared with that obtaining at parturition and the possible rôles of the ovarian hormones in

determining the alterations in the uterine muscle of the rabbit and of the human subject during pregnancy and parturition are discussed.

Certain considerations bearing on the clinical use of œstrin are suggested.

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A SIMPLE PHOTOELECTRIC COLORIMETER.

By G. A. MILLIKAN.

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Ordinary colorimetric methods of measurement suffer from the disadvantage of requiring subjective judgments depending on the colour sensitivity of the eye of the observer. Their accuracy is limited by the eye fatigue which they engender and is dependent upon the quality of the light by which they are made. Most of them are also subject to other limitations: they require the use of complex and empirical calibration curves, are relatively slow, or necessitate fairly large quantities of material. The improvements brought about by the use of spectroscopic or photometric methods are still limited by the basic disadvantage of subjective measurement, intensities of two fields having to be balanced against each other or spectral bands located. This paper describes a simple colorimeter, using a differential copper copper-oxide photoelectric cell and two colour filters, which possesses none of these disadvantages. It is relatively cheap, simple, and quick to use, can be adapted to very small quantities of fluid, and has a linear calibration curve. It is especially well adapted to the measurement of the degree of oxygenation of hæmoglobin solutions, and is not subject to the usual disadvantages of the other optical methods (such as inaccuracies due to inactive hæmoglobin) which have been used for this purpose.

Principle of the method.

The principle of the method can be seen from the diagram at the bottom of Fig. 1, which illustrates its applicability to the oxygenation reaction of hæmoglobin. Light from a suitable source passes through the absorption trough containing the solution being tested. Behind the trough two colour filters are fastened side by side in front of a differential photoelectric cell, so that part of the beam goes through each. The differential type of cell measures directly the difference in the amount of light falling on each half of its light-sensitive surface, and acts in effect

like two photoelectric cells working against each other. The two colour filters are so chosen as to match in a rough way the colours of the two end-points of the reaction being measured, so that when the colour goes from one extreme to the other there will be an increase in the amount of light going through one filter and a decrease in the amount going through the other. This reverses the distribution of light falling on the two sides of the cell, and with it the galvanometer deflection.

The working of the device is well illustrated by the hæmoglobin reaction for which it was specifically developed. As can be seen from the

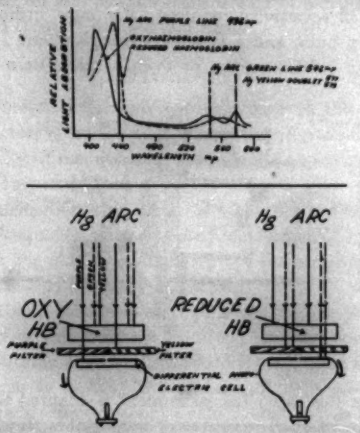

Fig. 1. Principle of photoelectric colour analyser applied to *HB*.

absorption curves plotted at the top of Fig. 1, the mercury arc is a peculiarly well-adapted source for this colour change. Violet light of wave-length 436 mμ, where there is a very strong emission line is very much more strongly absorbed by reduced than by oxyhæmoglobin, while the yellow and green lines (579, 576, and 546 mμ) are almost identical in wave-length with the peaks of the characteristic alpha and beta bands of oxyhæmoglobin, the position of which is used as the criterion for oxygenation by the Hartridge reversion spectroscope method. The Wratten D colour filter allows the violet light to pass almost undiminished and cuts out the yellow and green completely, while the yellow Wratten G filter allows these two colours to pass but is opaque to the violet. The

change from oxyhæmoglobin to reduced hæmoglobin in the absorption trough reverses the direction of the galvanometer current as shown in the diagram, partially oxygenated solutions producing intermediate deflections.

Description of the colorimeter.

A cross-section of the instrument is given in Fig. 2. It consists of light source, absorption trough, colour filters, photoelectric cell and galvanometer. The choice of source, trough, and galvanometer will depend upon the particular reaction being measured, the amount of material available, and the degree of sensitivity required. For most ordinary uses an incandescent lamp bulb, and a flat-sided trough about 1 cm. thick, with a low sensitivity galvanometer, or even a micro-ammeter, will prove

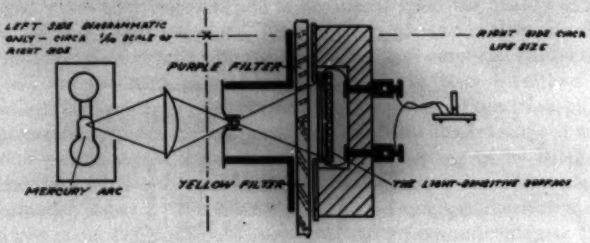
LEFT SIDE DIAGRAMMATIC ONLY — CIRCA ½ SCALE OF RIGHT SIDE
RIGHT SIDE CIRCA LIFE SIZE
PURPLE FILTER
MERCURY ARC
YELLOW FILTER
THE LIGHT-SENSITIVE SURFACE

Fig. 2.

satisfactory. As illustrated the instrument is adapted to measuring the oxygen saturation of small quantities of hæmoglobin, flowing down a tube. A KBE 110-volt mercury arc lamp is used, focused on the absorption trough with a condensing lens. The trough itself is a 1 cm. length of 1 mm. glass capillary.

The photoelectric cell is the differential type of copper copper-oxide cell manufactured by the Cambridge Instrument Co. under Westinghouse patents. It consists of a copper disc about 2 cm. in diameter, with a layer of copper oxide on the front surface, and a thin metallic film on top of this. The surface is divided into two halves by a scratch along one diameter, and the terminals rest lightly on the metallic film, one on each half. This cell measures directly, and without driving potential, the difference in the amount of light striking the two halves. The colour filters are fastened to the photoelectric cell. They are adjustable from side to side, enabling the galvanometer to be brought to a convenient

balance during calibration, and are then clamped fast. The adjustment is so made that the two end-points give deflections which are approximately equidistant from the null point on either side. This use of a balanced system very greatly simplifies operation, and reduces to a minimum the ill effects of fluctuations in light source and lack of mechanical rigidity in the apparatus. It has proved of the utmost importance in getting reliable results.

With this apparatus, using the 1 mm. observation tube, and the mercury arc source, I have obtained galvanometer currents of about 3×10^{-7} amperes for the change from 0 to 100 p.c. oxygenation of a hæmolysed blood solution diluted 200 times.

Calibration.

The galvanometer deflections corresponding to the two end-points are obtained directly either by successively running the fully oxygenated and the fully reduced hæmoglobin through the observation tube, or by using a three-compartment absorption trough, one compartment of which contains the unknown fluid, and the other two the end-point standards. The frequency with which these standards must be referred to depends upon the stability of the set-up, the steadiness of the lamp source, and the desired accuracy of reading.

The intermediate concentrations of the different pigments can be calculated by linear interpolation from the two end-points, when sufficiently dilute solutions, such as the method is well suited to, are used. The condition of linearity is that the amount of light absorbed by each substance be directly proportional to its concentration in the solution. In a parallel-sided trough, light absorption actually follows the well-known logarithmic expression:

Amount of light absorbed = amount of incident light $\times (1 - e^{-k \times c \times d})$,

where k is the specific light absorption of the substance, c its concentration, and d the thickness of the absorption trough. A simple calculation shows, however, that if a certain concentration absorbs 4 p.c. of the total light, then half that concentration will absorb 2·02 p.c. of the total light, which is only $\frac{0{\cdot}02}{2{\cdot}02} = 1$ p.c. deviation from linearity. If an accuracy of 1 p.c. is desired, it is then necessary to see that the galvanometer deflection between end-points is less than 4 p.c. of the total deflection obtained by cutting off completely the light striking one side of the cell.

If this dilution condition has been complied with, the calibration curve is a straight line. To understand this, we need only make the

assumption, which has been often made before, and is in fact implicit in the calibration of the reversion spectroscope by the wedge trough method, that a partially oxygenated solution of hæmoglobin behaves optically like a mixture of the corresponding quantities of oxy- and reduced hæmoglobin. Let us, for the sake of simplicity, consider the case of a solution 50 p.c. saturated. The absorption curve of such a mixture will lie just half-way between that of the fully oxygenated and that of the fully reduced one given in Fig. 2, and in particular will cut the violet, green and yellow lines of the mercury arc spectrum just half-way between the intercepts of the two extreme curves. The violet light striking one side of the photoelectric cell will then be of an intensity mid-way between that of the two end-point solutions, and the same will be true of the yellow and green light striking the other side of the cell. Since the galvanometer records the algebraic sum of these different effects, its deflection will also be just half-way between its two calibrating end-point positions. Similarly, a solution one-third saturated will produce a galvanometer deflection one-third of the way from the fully reduced to the fully oxygenated positions, and so on.

The argument applies equally well to every reaction which involves any colour change, *i.e.* any alteration in the spectral absorption. In particular, it is applicable to mixtures of reduced hæmoglobin and carboxy-hæmoglobin; and at Dr Roughton's suggestion, I have used these mixtures to test empirically the linearity of the calibration curve, since known mixtures of reduced and carboxy-hæmoglobin are very much simpler to prepare than those of reduced and oxyhæmoglobin. The affinity of carbon monoxide for hæmoglobin is so great that one can make up a solution of saturated carboxyhæmoglobin, and subsequently remove practically all of the excess carbon monoxide without danger of appreciable dissociation. Such a solution was mixed in varying proportions with a solution of hæmoglobin previously reduced by bubbling nitrogen through it and adding a small quantity of sodium hydrosulphite, and its satura-

Actual p.c. COHb	Measured p.c. COHb photo-cell method	Maximum deviation
20	21	4
40	38	4
60	60	3
80	80	8

tion was then measured by the photoelectric cell method. The average of four determinations for each solution are given in the above table, as well as the maximum deviations of individual readings.

By this rough test the method appears accurate to about 4 p.c. for individual readings. The measurements were made under the very unfavourable conditions imposed by an absorption trough consisting of a cylindrical tube less than 2 mm. in internal diameter. This type of trough was dictated by the nature of the kinetic studies for which the method was being developed. With a larger observation vessel, and more light passing through it, a much higher degree of accuracy should be obtainable.

The linear character of the calibration curve is one of the principal advantages of the method. It reduces the labour of calculation and insures uniform accuracy throughout the whole colour range. It is, in this respect, more satisfactory for hæmoglobin reactions than the reversion spectroscope, which has an empirical calibration curve, and which becomes unreliable at both high and low degrees of saturation.

SUMMARY.

A colorimeter using a differential copper copper-oxide photoelectric cell, and colour filters is described. Its advantages are:

(1) Elimination of eye strain and subjective judgments.
(2) Speed and ease of operation.
(3) Linear calibration curve.
(4) Availability for small quantities of material.
(5) Simplicity and low cost.

It has been used to measure the degree of oxygen saturation of dilute hæmoglobin solutions.

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THE KINETICS OF BLOOD PIGMENTS: HÆMOCYANIN AND HÆMOGLOBIN.

By G. A. MILLIKAN.

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Introduction.

Oxygen unites reversibly with the respiratory pigments, hæmocyanin and hæmoglobin. When the equilibrium state has been reached, as many molecules of oxygen are being bound per second by the pigment as are dissociating from it. This paper describes the individual measurement of these two opposing reactions oberved in several kinds of hæmocyanin and hæmoglobin. It follows directly both in method and subject-matter the pioneer researches of Hartridge and Roughton [1923 *b*, 1925] on the kinetics of sheep hæmoglobin.

Measurements of the speed of the reactions of oxygen with the respiratory pigments can throw light directly on two groups of questions: (1) Is the speed with which oxygen is taken up or given off by the blood pigments slow enough to be a physiological limiting factor in the metabolism of the animal? (2) What is the mechanism by which the blood pigments bind oxygen? It is also hoped that these studies on the rates of reaction of the small oxygen molecules with large protein molecules may eventually throw some light on the mechanism of enzyme action, and serve as an introduction to a later kinetic study of catalytically active pigments.

I. Method and material.

The experimental method.

The continuous flow method for following rapid reactions taking place in solutions, which was introduced by Hartridge and Roughton [1923 *a*], has been used. The two reacting fluids are driven at a high rate of speed into a common chamber where they are thoroughly mixed, and immediately passed down an observation tube, where the colour changes accompanying the reaction can be followed, each point along the

observation tube corresponding to a fixed calculable time after the initial mixing. The apparatus described by Hartridge and Roughton had two principal limitations which have been overcome in the present form. First, it was only adapted to relatively large amounts of material, which were not available when hæmocyanin or hæmoglobin from small animals was being studied. This has been remedied by constructing the device on a micro scale, the observation tube being a little less than 1 mm. in internal diameter. Second, the reversion spectroscope with which Hartridge and Roughton determined the degree of oxygenation of the hæmoglobin is not available for hæmocyanin, while even for hæmoglobin it can only give readings between about 25 and 75 p.c. saturation, thus missing the very important initial and end-points of the curve. Photoelectric cell methods have accordingly been developed, which are not subject to these weaknesses. The increase in total light absorption which accompanies the oxygenation of hæmocyanin when it goes from colourless to blue was used as the criterion for the extent of the reaction when this substance was being studied, while for hæmoglobin the change in relative absorption of different wave-lengths, which is indicated by the purple-to-scarlet shift, was measured. One such method is described in an adjoining paper [Millikan, 1933], and it is hoped in due course to discuss the remainder of the technique elsewhere.

In accordance with the procedure of Hartridge and Roughton the dissociation process has been measured by removing the oxygen liberated by the reaction as fast as it is formed by means of sodium hydrosulphite. This reduces the back reaction to zero. In measuring the rate of combination we have limited ourselves to the first portion of the reaction, where the backward component is negligible or can easily be allowed for. In the reduction process, a solution of oxygenated pigment is driven in one entrance of the mixing chamber and a solution of hydrosulphite in the other, the change of colour in the observation tube being then measured. It was necessary, however, to show that the oxygen absorber actually does act in this capacity, and does not attack directly the molecules of oxygenated pigment. By varying the initial hydrosulphite concentration, Hartridge and Roughton were able to show that in the reduction of hæmoglobin the reagent did act in this desired way, for, after a certain minimal concentration was reached, no further increase produced a change in the velocity of dissociation. I have been able to confirm this result for hæmoglobin, and to extend it to hæmocyanin. Furthermore, I have been able to show that the behaviour is completely different where hydrosulphite acts as a true reducer as in the reduction of methylene blue

to leuco methylene blue or of methæmoglobin to reduced hæmoglobin, and for these two substances the rate of reduction, as measured by the reciprocal of the time required to reach half completion, is directly proportional to the concentration of reducer, as one would expect from the simplest mass action considerations (see Table I, and Fig. 1).

TABLE I. Effect of hydrosulphite concentration on dissociation rate.

Substance and date	Time for half reduction (sec.) 0·2 p.c. $Na_2S_2O_4$	0·4 p.c. $Na_2S_2O_4$
Deoxygenation		
Limulus hæmocyanin, raw, Aug. 1931	0·065 ±0·01	0·065 ±0·01
Limulus hæmocyanin, dialysed, Aug. 1931	0·075 ±0·01	0·075 ±0·01
Limulus hæmocyanin, raw, Apr. 1932	0·092 ±0·01	0·082 ±0·01
Maia hæmocyanin, raw, Feb. 1931	0·013 ±0·005	0·010 ±0·005
Human hæmoglobin, July 1932	0·017 ±0·002	0·014 ±0·002
True reductions		
Methylene blue, Nov. 1930	0·052 ±0·01	0·023 ±0·005
Human methæmoglobin, July 1932	0·053 ±0·012	0·025 ±0·010

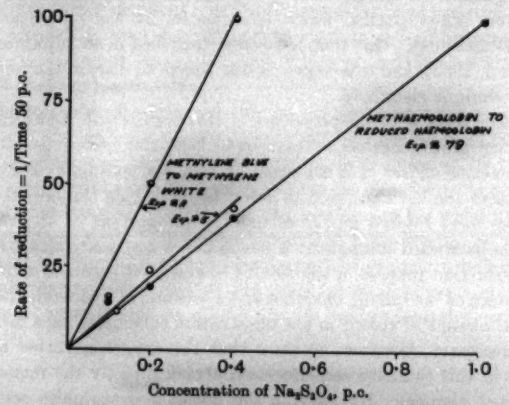

Fig. 1. Effect of reducer concentration in true reductions.

The control of the *p*H required special experimental precautions, because the sodium hydrosulphite used as an oxygen absorber acts as an acid in solution, and its oxidation products are more acid than it is. The reducing solutions of hydrosulphite were therefore made up in *M*/20 borate or phosphate buffers, with a small measured excess of NaOH added to bring back the reaction to that of the original buffer. Indicators could not be used to determine the *p*H with accuracy because of the bleaching effect of the hydrosulphite, so the reaction was in all cases determined with the glass electrode, and a large number of pre-

liminary tests were made to show that the final equilibrium *p*H was the same as that reached immediately after mixing in the observation tube. Since it was found that the effluent solutions became more acid on standing in the air, it was necessary to make the *p*H determinations during or immediately after a run.

In order to eliminate possible gradual changes in the pigments at different *p*H's, it was the usual procedure to use as stock solutions in the apparatus a single batch of unbuffered pigment on the one hand, and on the other a series of buffered reducer solutions of different reactions.

Substances used.

Three principal respiratory pigments have been used in this study:

(1) The hæmocyanin of the horseshoe crab, *Limulus polyphemus*, dialysed, and also in the native serum.

(2) The serum of the spider crab, *Maia squinado*.

(3) Laked sheep blood in dilute solution.

The first substance, in the purified form, is the best-established example of a "primitive" oxygen carrier, *i.e.* one for which the equilibrium dissociation curve is reproducibly a simple hyperbola. Its behaviour is further simplified by the fact that this curve is relatively insensitive to changes in hydrogen-ion concentration [Redfield, 1930]. *Maia* serum, on the other hand, represents a pigment which has "gone wild." Its equilibrium curve is of variable sigmoid form, and is enormously affected by *p*H. It has a very marked minimum affinity at *p*H 6·2, and large increases on both acid and alkaline sides of this reaction [Hogben, 1926]. The native serum of the horseshoe crab has properties intermediate between these two extreme groups, the equilibrium curve being generally sigmoid in shape, and quite definitely affected in position by change of reaction, though not nearly as much so as that of the Crustacea. There is also a marked minimum affinity at a *p*H of 8·1 [Redfield, 1931].

Laked sheep blood was chosen for comparative study because it represents a hæmoglobin whose equilibrium curve has been very carefully worked out, and because it was the object of the earlier kinetic studies of Hartridge and Roughton with whose results the new data could therefore be compared. Some kinetic experiments have also been made on the hæmoglobins of man, frog, and pig, in order to compare the kinetic behaviour of a number of hæmoglobins with each other. The development of the micro method has for the first time made possible this type of investigation on the smaller or the more valuable animals (viz. frog, man), whose blood is only available in limited quantities.

Limulus serum, treated with toluol to prevent bacterial action, will keep many months without deterioration if stored at 0° C. I am extremely grateful to Dr A. C. Redfield for having generously provided me with a large amount of this material from Wood's Hole, Massachusetts. He drew the blood, filtered it through cheesecloth, added toluol, and shipped

it to me in the ice-boxes of fast liners. Handled in this way, the material remained in apparently good condition. Dialysis was carried out in the usual collodion sacks against distilled water at about 0° C., until the chloride content became less than 1 millimolar as judged by the amount of precipitate formed when an excess of silver nitrate was added to a nitric acid digest of the sample. Control tests were made with known salt solutions.

The formation of melanin proved extremely troublesome in the spider crab serum [Pinhey, 1930], *Limulus* blood being fortunately free of the tyrosinase which produces it. This tendency to blackening, the occasional presence of yellow lipochrome pigments, and the very great variation in the quality and concentration of pigment in spider crab blood made it a very much less satisfactory substance to work with than that of *Limulus* and reduced the accuracy of the results obtained with it. None of the devices used to combat these bad properties of the blood has been completely satisfactory.

Sheep hæmoglobin was prepared as described by Forbes and Roughton [1931] or Hartridge and Roughton [1923 *b*], in order that the results might be comparable with those obtained in earlier work. Human hæmoglobin was obtained a few minutes before each experiment by defibrinating and laking with distilled water a few cubic centimetres of blood drawn from an arm vein. Two or three c.c. of red blood were sufficient for an entire kinetic run with thirty or forty individual points, but the poorer optical properties of hæmocyanin require for it greater concentration, so that 50–75 c.c. of the blue blood were usually necessary.

Calculation of velocity and equilibrium constants.

In calculating the velocity and equilibrium constants for "primitive" pigments, the following expressions were used:

(1) K = equilibrium constant $= \frac{1}{p_{O_2} \text{ at 50 p.c. saturation}}$;
Dimensions: $\frac{1}{\text{mm. Hg}}$.

(2) $K = \frac{k'}{k} = \frac{\text{association velocity constant}}{\text{dissociation velocity constant}}$.

(3) $k' = \frac{1}{a \times t_{50}}$ = association velocity constant;
a = initial concentration of both oxygen and reduced hæmocyanin in mm. Hg of O_2;
t_{50} = time of half completion of reaction;
Dimensions: $\frac{1}{\text{mm. Hg} \times \text{sec.}}$.

(4) $k = \frac{0{\cdot}7}{t_{50}}$ = dissociation velocity constant;
Dimensions: $\frac{1}{\text{sec.}}$.

These expressions are a simpler and somewhat less accurate version of those given by Hartridge and Roughton [1925]. The following assumptions have been made in deriving them:

(*a*) The dissociation is monomolecular.

(*b*) In measuring the dissociation rate, the hydrosulphite absorbs the oxygen so quickly that there is no back reaction.

For these first two assumptions the evidence, both old and new, is now very strong. They should be valid quite independently of the validity of the remaining three.

(*c*) The association is bimolecular.

As far as the new kinetic studies go, the hyperbolic shape of the equilibrium dissociation curve is our only justification for making this assumption.

(*d*) In measuring the association rate, the back reaction is negligible.

(*e*) In measuring the association rate, the initial concentration of oxygen and reduced pigment are equivalent.

These last two assumptions can be checked in the following way. The complete differential equation for the association process is

$$\frac{dy}{dt} = k'(a-y)(b-y) - ky,$$

where y = concentration of oxygenated pigment,
a = initial concentration of oxygen,
b = initial concentration of reduced pigment.

This expression was given by Hartridge and Roughton. It can be integrated graphically upon substitution of any arbitrary values for k', k, a, and b. If assumptions (*d*) and (*e*) are made this equation becomes

$$\frac{dy}{dt} = k'(a-y)^2,$$

which can very easily be integrated. Assumption (*d*) was checked by comparing the time of half completion of the reaction as calculated by graphical integration of these two equations, the substituted values being those of actual kinetic runs on *Limulus* hæmocyanin. The difference was just 10 p.c., which is less than the experimental uncertainty in the determination of this quantity. The final assumption (*e*) depends upon the experimental conditions. It was not always possible to make the concentration of the reactants exactly equivalent, but an excess of either one amounting to as much as 40 p.c. can be shown by integration of the corresponding equations to affect the half completion time of the reaction by less than 35 p.c., which is about the experimental uncertainty in this quantity.

The concentration units in which all of the constants are expressed is that in common use for equilibrium curve work, namely, "the equivalent partial pressure in millimetres of mercury." The reduced and oxygenated pigments as well as the dissolved oxygen can be very simply measured in this way, the pigment concentration being expressed in terms of its oxygen capacity. Roughton [1932] has given the thermodynamic

justification for the unit. It is used here because of its convenience, but it can readily be converted into the usual chemical notation by means of the Bunsen solubility coefficient and the gas constant.

It has been pointed out by Adair and by Hartridge and Roughton that the same kinetic and equilibrium equations are obtained if the pigment molecule contains any number of places for oxygen instead of only one, provided that these valencies are all equally strong and are independent of each other. For purposes of calculation one "molecule" of the pigment would, however, be defined as the amount of substance combining with one molecule of oxygen.

II. Experimental results.

Under physiological conditions.

The direct physiological interest in kinetic experiments on the blood pigments lies in the answer to the question: are these reactions slow enough to be limiting factors in the metabolism of the animal? In the vertebrates the question is complicated by the fact that here the pigment is carried in corpuscles, diffusion and chemical reaction velocity becoming joint factors in determining the total rate of gas exchange, as has been shown by Roughton [1932]. Hæmocyanin, however, is dissolved directly in the blood, and this complication does not exist in its case. *In vitro* experiments on hæmocyanin therefore give us a much more direct picture of what goes on in the organism than corresponding ones on hæmoglobin. In two other respects the experimental conditions in the kinetic studies of the blue bloods approach more closely those obtaining in the living animal. Due to optical properties of the pigments, the concentration of hæmoglobin must be reduced to one-hundredth or less of that in the corpuscles in order to make kinetic measurements, and it is conceivable that this great dilution may considerably affect the measured velocity constant of the reaction. Hæmocyanin, however, can be studied at practically blood concentrations. In the second place, normal room temperature, at which these experiments can most easily be carried out, is a normal one for the blue-blooded animals, but is below the body temperature of the mammals.

Table II summarizes the results of kinetic dissociation runs made on the native sera of the spider crab, *Maia*, and the king crab, *Limulus*, at room temperature and at hydrogen-ion concentrations in the neighbourhood of the physiological range [Quagliarello, 1916]. In Fig. 2 are plotted the relevant points of two of the individual experiments. Several

TABLE II. Rate of dissociation of arthropod sera under physiological conditions.

Exp.	Species and date	pH	Time, 50 p.c. reduction sec.
50	*Maia*, Nov. 1931	7·4	0·017 ±0·005
51	„ Nov. 1931	7·5 (interpolated)	0·020 ±0·008
52	„ Nov. 1931	7·5 (interpolated)	0·015 ±0·008
71	„ June 1932	7·5	0·020 ±0·004
	Average, *Maia*, *c.* 20° C.		0·017 ±0·006
46	*Limulus*, Aug. 1931	?	0·065 ±0·020
56	„ Feb. 1932	9·0	0·075 ±0·010
57	„ March 1932	8·0	0·075 ±0·010
59	„ April 1932	8·0	0·075 ±0·010
60	„ April 1932	8·0	0·080 ±0·015
	Average, *Limulus*, *c.* 20° C.		0·075 ±0·010

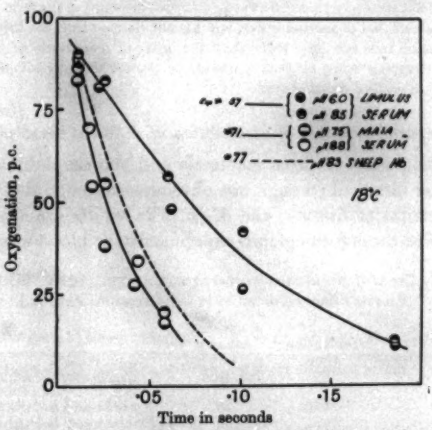

Fig. 2. Rate of dissociation of sera; physiological conditions.

of the points are averages of two or more readings to avoid congestion in plotting. A corresponding curve for sheep hæmoglobin is included for reference. It will be seen that the rate at which oxyhæmocyanin dissociates is of the same general order as that of oxyhæmoglobin, the half reaction taking from a sixtieth to a tenth of a second. There is, however, a very considerable difference between the two hæmocyanin species, the relatively active spider crab possessing blood which yields up its bound oxygen much more rapidly than the more sluggish horseshoe crab. It is improbable that this difference is of any physiological significance, since

even the most slowly dissociating pigment gives up half its oxygen in one-tenth of a second, whereas the time of passage of blood through the tissue capillaries is never less than several seconds.

The rate of oxygen uptake at physiological concentrations is a much quicker affair than the reverse process, reaching half completion in less than 1/300 second for both *Limulus* and *Maia* serum, which is very much too fast to make it a limiting factor in the metabolism of the animal.

The observation tube of the kinetic apparatus does not give a strictly accurate picture of what takes place in the gill capillary, since in the former the oxygen is used up from the supply initially sent down the tube with the reduced pigment, and there is therefore a progressively lower oxygen tension as we proceed along the tube; in the intact animal, on the other hand, oxygen is drawn from outside the capillary, where it exists at a fairly constant concentration. In the kinetic experiments, the initial oxygen tension of about 300 mm. of Hg was chosen so that the average oxygen pressure as the fluid went down the tube would be about that of aerated water, but it is not claimed that the value of 1/300 sec. for half completion tells anything more than the order of magnitude of the rate in the capillary. This happens to be all that is needed to answer the physiological question of limiting factors in oxygen metabolism.

The comparative speeds of dissociation of different blood pigments.

The hæmoglobins of three mammals and one amphibian have been studied under identical (though not physiological) conditions, as well as the hæmocyanins of *Limulus* and *Maia*. The results are summarized in Table III. For these hæmoglobin experiments the blood was hæmolysed

TABLE III. Rates of dissociation compared with oxygen affinity. *p*H 8·6, 22° C. (Kinetic values all obtained in same experimental run.)

Hæmoglobins	Reduction time t_{50}	Reciprocal affinity p_{O_2} – 50 p.c. sat. (mm.)	Authority for affinity values
Frog	0·020	18	Macela and Seliškar [1925]
Sheep	0·028	3	Forbes and Roughton [1931] / Hartridge and Roughton [1923*b*]
Man	0·038	1	Macela and Seliškar [1925]
Pig	0·047	Unknown, but low	
Hæmocyanins			
Spider crab, *Maia*	0·025	16	Hogben [1926]
King crab, *Limulus*:			
Dialysed	0·080	3	Redfield [1930]
Native	0·075	17	Redfield [1930]

and diluted two hundred times in borate buffer. No attempt was made to remove the stromata, salts, or plasma proteins, but their concentration would be low in the final dilute solution. In any case, the effect of salt concentration on the rate of dissociation is probably not large, as its

removal is seen to have little effect on this quantity in *Limulus* hæmocyanin (Table III). The value for *Maia* hæmocyanin given in the table is for the native serum, as no extensive runs were made on this material after dialysis. In Table III are also given the partial pressures of oxygen in equilibrium with half-saturated pigment solutions, as a rough measure of the oxygen affinity. Their significance will be discussed in a later section.

The order of the dissociation reaction.

The kinetic dissociation curve of both hæmoglobin and hæmocyanin has been found to be monomolecular within the limits of error of the

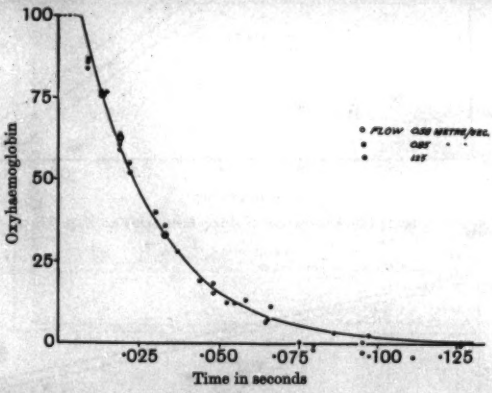

Fig. 3. The dissociation of sheep hæmoglobin. Exp. 77, *p*H 8·3. Curve constructed from equation $y/100 = \rho^{-44.5(t-0.0074)}$; reduction velocity constant $= 44{\cdot}5 \frac{1}{\text{sec.}}$; time of induction $= 0{\cdot}0074$ sec.

experiments, quite independent of the shape of the equilibrium dissociation curve. The data of one experiment on sheep hæmoglobin are plotted in Fig. 3, and replotted logarithmically in Fig. 4. These findings completely confirm the earlier results of Hartridge and Roughton obtained with the reversion spectroscope on the same material, and they furthermore extend the measurements to much higher and lower saturation ranges where the test for monomolecularity becomes more severe. The standard deviation from the logarithmic curve of all points above 3 p.c. saturation is 3·3 p.c. (This is raised to about 5 p.c. if we include the points on the extreme tail of the curve, which were thrown somewhat low by a systematic error of manipulation.) The mutual consistency of points

made with widely differing rates of flow gives satisfactory evidence that these results have a real meaning and are not experimental artefacts.

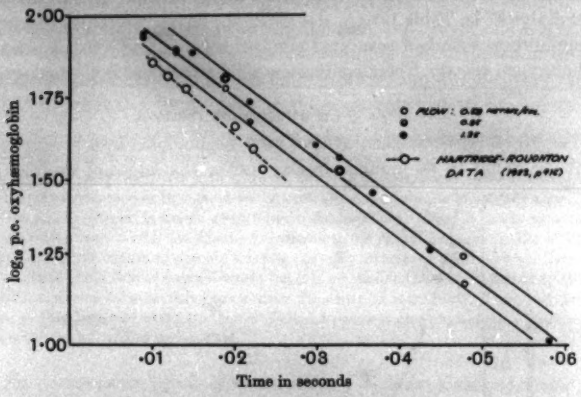

Fig. 4. The order of the dissociation of sheep hæmoglobin. Exp. 77, *p*H 8·3.

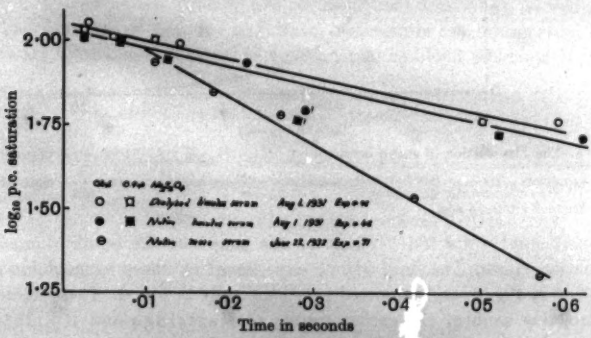

Fig. 5. The order of dissociation of hæmocyanin.

Much less accuracy is obtainable with hæmocyanin, but within the wider limits of experimental error, both *Maia* and *Limulus* sera show the same monomolecular dissociation curves (see Fig. 5), the logarithm of the amount of oxyhæmocyanin decreasing uniformly with increasing time. This is true for *Limulus* serum both before and after dialysis, though the

equilibrium curve is much affected in shape and position by this purification. Neither the shape nor the position of the kinetic dissociation curves is affected by a two- or threefold change in the concentration of hæmocyanin (*Limulus*).

Effect of pH on dissociation rate.

The effect of varying the *p*H on the rate of oxygen dissociation differs very considerably from substance to substance. The shifts will be most easily understood in relation to the corresponding changes in equilibrium behaviour, for which the relevant data have been summarized in Fig. 6.

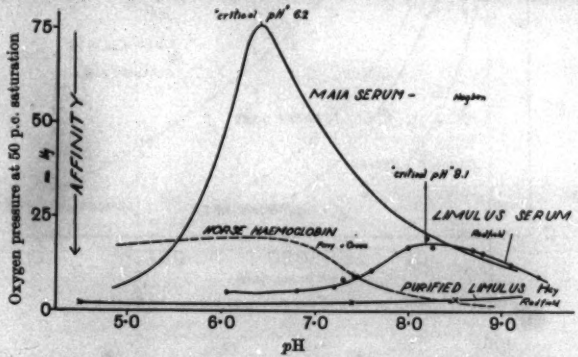

Fig. 6. *p*H effect on equilibrium curves: hæmocyanin and hæmoglobin.

It had been previously found by Hartridge and Roughton that the dissociation rate of dilute sheep hæmoglobin was much higher in acid than in alkaline solution. By repeating their experiments on the new apparatus, I have been able fully to confirm their results, and to show that they can also be carried over to human material (Fig. 7). As was expected from the greater affinity of human hæmoglobin for oxygen [Forbes and Roughton, 1931], the rate of dissociation was in every case considerably lower than that of the sheep, but the character of the change with *p*H was found to be the same. If the velocity constant is plotted against *p*H, the curve is seen to resemble the titration curve of a monovalent acid with a *p*K of about 7, there being a low constant rate at the alkaline end, a high constant rate at the acid end, and an intermediate transition portion.

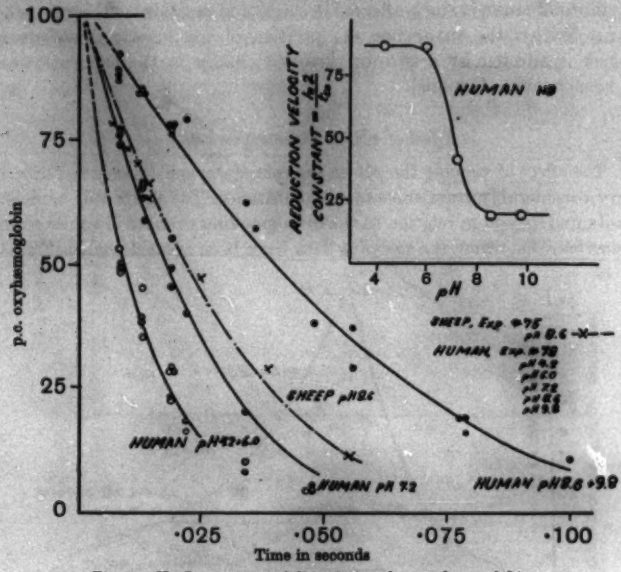

Fig. 7. *p*H effect on rate of dissociation: human hæmoglobin.

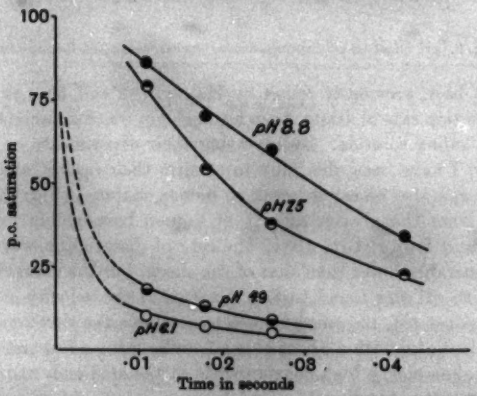

Fig. 8. *p*H effect on rate of dissociation: *Maia* serum. 19° C. Exp. 71. Native serum.

The hæmocyanins of *Limulus* and *Maia* show completely different effects, *Maia* being very sensitive indeed and *Limulus* relatively insensitive (Figs. 8, 9). This reflects the general equilibrium behaviour plotted in Fig. 6. It will be seen that the rate of dissociation of *Maia* serum increases enormously as the solution becomes more acid, but there is no marked slowing up or "come back" in the rate after the critical *p*H (6·2) has been passed, although the oxygen affinity does show such a marked return. *Limulus* serum, on the other hand, shows only a small *p*H effect, the rate increasing slightly with acidification. Here also there

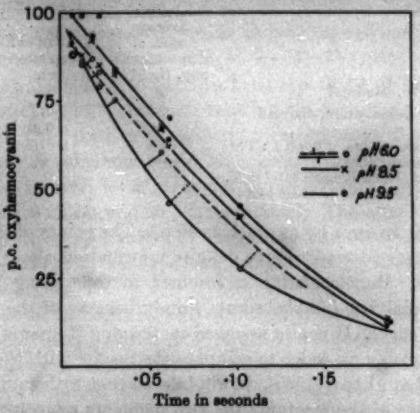

Fig. 9. *p*H effect on rate of dissociation: *Limulus* serum. Exp. 57. Points are individual readings.

are no signs of a return to lower speeds on the acid side of the "critical *p*H" (8·1). The *p*H effect on this species is, however, so small as to be within the rather wide limits of experimental uncertainty. No satisfactory experiments on the *p*H effect of the dialysed *Limulus* material have been made.

Effect of temperature on the dissociation rate.

Increasing the temperature greatly speeds up the dissociation rate of purified *Limulus* hæmocyanin, just as Hartridge and Roughton found it to do for sheep hæmoglobin. The experimental uncertainties in the hæmocyanin temperature work were, however, too great to allow an exact calculation of the temperature coefficient. The value of Q_{10} is probably

about 3·8 (the same as the hæmoglobin value), though one experiment gave as low as 2·2. The shift in the equilibrium constant for this material has been found by Brown [1933] to have a Q_{10} of 2·3.

The rate of combination of oxygen with reduced pigment.

The new experiments have not yielded quite as accurate results for the speed of oxygen association of hæmocyanin as Hartridge and Roughton were able to obtain for sheep hæmoglobin, the values given in Table IV

TABLE IV. Rate of combination of oxygen and reduced pigment.

Exp.	Material	Initial average conc. of reactants mm. Hg of O_2 a	Time of half completion sec. $t_{\frac{1}{2}}$	Association velocity constant assuming bi-molecularity k'
67	*Limulus* hæmocyanin, dialysed	30	0·012	2·8
68	" " "	32	0·008	3·9
65	Sheep hæmoglobin, diluted 1 : 200	25	0·010	4·0
	Sheep hæmoglobin Hartridge and Roughton [1925, p. 674], converted to these units	—	—	4·5–6·3

being possibly in error by as much as 40 p.c. The points of the individual curves are too scattered to give definite information about the order of the reaction. Bimolecularity is assumed in calculating the velocity constant of dialysed *Limulus* serum simply because of the shape of the equilibrium curve. It is also assumed in deriving the same constant for sheep hæmoglobin, in order to compare the results with those of Hartridge and Roughton [1925]. It will be seen that with equivalent initial concentrations of the reactants the two pigments react with oxygen at roughly the same rate of speed.

In order to catch the association reaction at all, a redesigned form of the kinetic apparatus was required, which would allow greater dilutions to be used. This apparatus has an observation tube 2 mm. in diameter (instead of the usual 1 mm. tube), and a 4-jet mixer (instead of the usual 2-jet type). No other changes were made in the experimental arrangements.

III. DISCUSSION OF RESULTS.

The relation of "on," "off," and equilibrium constants.

The purified hæmocyanins of *Limulus*, *Helix*, and *Busycon* are the only respiratory pigments now known which reproducibly give hyperbolic equilibrium curves and for which, therefore, the very simple Hüfner assumptions can reasonably be made. One of these substances is the

logical choice for a comparison of the observed equilibrium affinity with that calculated from the measured rates of the two opposing kinetic reactions. These values for dialysed *Limulus* hæmocyanin are given in Table V.

TABLE V.

Association		Dissociation		Equilibrium	
					Observed
Exp.	k'	Exp.	k	Calc.	[Redfield]
67	2·8	69	7·4	0·38	0·40
68	3·9	—	—	0·53	—

Exps. 67, 68, and 69 were all made on the same material and on adjacent days. The "observed" value of 0·40 for the equilibrium constant is interpolated from the data of Redfield [1930], and Brown [1933]. I have been able to confirm both the shape of the curve and the value for the equilibrium constant by these workers, on material identical with that used in the kinetic measurements, though the uncertainty of my K value was considerably greater than theirs, being about 0·1. The possible errors for k', k, and K as judged from the experimental uncertainties are 20, 50, and 25 p.c. respectively. The agreement between observed and calculated values of the equilibrium constant is therefore as good as could have been expected, being within 5 p.c. in one case and 35 p.c. in the other.

The situation in regard to hæmoglobin is much less satisfactory. Hartridge and Roughton [1925] obtained about the same agreement between the velocity and equilibrium constants on sheep hæmoglobin as have now been obtained on *Limulus* hæmocyanin. They had determined equilibrium curves on this material, using the reversion spectroscope, and had found them to be hyperbolic in shape. Later investigation, however, especially by Forbes and Roughton [1931], has reproducibly given sigmoid curves for dilute solutions of sheep hæmoglobin. The simple Hüfner theory is no longer able to provide an accurate description of these experimental facts, and we must seek agreement between kinetic and equilibrium data on the basis of other primary assumptions.

Kinetic data can be of help in assigning relative merit to other theories of the nature of the oxygen-respiratory pigment complex, although the quantity and accuracy of the kinetic results so far obtained are not sufficient to give a definitive award to any one. The Hill theory, which has generally been regarded as untenable in its original form since the two independent determinations of Adair and Svedberg, which gave agreeing values for the molecular weight very different from that predicted by this hypothesis, also fails in its prediction of kinetic behaviour of sheep

hæmoglobin in dilute solution. The kinetic equations given under "Method" for a simple pigment in which $n = 1$, can very easily be generalized for any integral values of n, and the velocity constants can be obtained in the same way. The very careful equilibrium curves of Forbes and Roughton, made under conditions which were duplicated as closely as possible in the present kinetic studies, have inflections which give n values between 2 and 3. The equilibrium K calculated from k' and k differs, however, from that obtained directly from the equilibrium curve, being five times too small for $n = 2$ and twenty times too small for $n = 3$. The combined possible errors, though fairly large, are very much less than this discrepancy. Since all values of n greater than 1 give completely unacceptable calculated values for the equilibrium constant, a theory based on mixtures of two kinds of such molecules (in which, for example, one is of the $n = 2$ form, and the other of the $n = 4$ form) such as Redfield has used in interpreting equilibrium curves, would have great difficulty in explaining the results of the kinetic measurements.

There remains the intermediate compound hypothesis suggested by Adair. This theory adequately accounts for the shapes of the carefully made equilibrium curves of Adair [1925], Ferry and Green [1929], and Forbes and Roughton [1931], if the four arbitrary constants predicted by it are suitably chosen. Its principal disadvantage, as a theory, is that it allows too much latitude in the choice of the constants. This lack of specificity is so great that until more evidence is available on the probable values of some of these constants, the attempt to correlate the kinetic and equilibrium data is perhaps futile. At this stage, however, two considerations do deserve emphasis:

(*a*) Analysis shows that it is quite possible for the intermediate compound hypothesis to lead to an accurately monomolecular curve for the dissociation velocity, even if the equilibrium curve is sigmoid (*e.g.* $n = 2$ to 3 on Hill's notation). It is hoped later to develop this matter more fully.

(*b*) Preliminary experiments by Roughton have shown that, under some conditions, there may be a difference between the "slow equilibrium" finally reached when oxygen and hæmoglobin are shaken together in tonometers, and the "quick equilibrium" reached in a fraction of a second when oxygen solutions and hæmoglobin solutions are mixed together in the rapid reaction apparatus. The photoelectric methods are particularly suitable for following up this point further. If it were indeed established it would obviously be of prime importance in the correlation of kinetic and equilibrium data.

The molecular weights of blood pigments.

We have seen how similar in their kinetic properties are hæmoglobin and hæmocyanin, and the equilibrium work of the last few years has but emphasized the essential likeness of these two groups of substances in their behaviour towards oxygen. The differences between the two groups are in general no greater than interspecies differences within each group. It is appropriate, therefore, to ask of what significance is a thirty- to seventyfold difference in their molecular weights, Svedberg's ultracentrifugal determinations having given a value of 5,000,000 for the weight of *Helix* hæmocyanin and 2,000,000 for *Limulus* hæmocyanin as compared with 68,000 for mammalian hæmoglobins. Adair's intermediate compound hypothesis is as easily capable of explaining sigmoid dissociation curves with $n = 30$ or $n = 70$ as with $n = 4$. This theory further suggests a very simple qualitative explanation for the observed change in shape of the curves under different conditions of salt concentration, for if the relative dependence of one oxygen valence on another is determined by the salt concentration, such changes are predicted. For example, in the case of *Limulus* hæmocyanin, the hyperbolic curve obtained for the purified material is predicted by the intermediate compound hypothesis if the tightness with which one oxygen molecule is bound in no way affects the equal tightness with which its neighbour is bound. This will be so no matter what is the molecular weight, and will also explain the indifference of the hyperbolic equilibrium curve of purified *Helix* hæmocyanin to its molecular weight, the Stedmans having shown that the position and shape of the curve are practically independent of the reaction between *p*H 4·0 and 9·0, whereas Svedberg has found the "5,000,000" molecules to be stable only between *p*H 4·5 and 7·5, beyond which they break up into smaller fragments. The addition of salts must now be supposed to introduce restricting influences on the oxygen valencies, so that they become to a certain extent dependent on one another. The altered equilibrium constants when substituted into the intermediate compound equation for oxygen equilibrium will now give it an S-shape, such as that observed experimentally.

Although Adair's theory was put forward on the basis of definite molecular weight data, it is now seen that its usefulness persists even in the absence of the support furnished by the molecular weight, and molecular size becomes of relatively little importance in fitting the oxygen data, so long as one molecule has at least several places for oxygen. It is quite possible that the giant hæmocyanin molecule is made up of

hæmoglobin-like "sub-molecules," in each of which $n = 4$ or some other small number, but so far we have no experimental evidence either for or against this hypothesis.

The equilibrium condition.

When a solution of hæmocyanin or hæmoglobin is in equilibrium with a given partial pressure of oxygen, just as many molecules of oxygen are being bound by the pigment every second as are dissociating from it. If any change in conditions produces an increase in the equilibrium affinity, this can only mean that there has been an increase in the rate at which oxygen molecules have been joining on or a decrease in the rate with which they have been coming off, or both. This is true quite independently of any theory of the mechanism by which they are bound. Hartridge and Roughton found that for sheep hæmoglobin it was the dissociation reaction which bore the brunt of such a change, the joining on process being little if at all affected by changes in *p*H, salts, or temperature. The experiments on hæmoglobins of several animals described in Section II show that qualitatively at least this principle may be extended to interspecies differences, the higher the affinity, the slower being the dissociation (Table III). There are difficulties, however, when we attempt to apply the same principle to the two hæmocyanins which have been investigated. The native hæmocyanins of *Maia* and *Limulus* at *p*H 8·6 have approximately equivalent affinities, but their dissociation rates differ by a factor of three. Furthermore, the large increase in the affinity of *Limulus* serum for oxygen brought about by dialysis fails to be reflected in any considerable change in the rate of dissociation. Another exception is found in the *p*H behaviour of both types of blue blood, for on the acid side of the "critical *p*H" acidification brings increased affinity for oxygen, but fails to bring marked slowing down of the dissociation process.

Two possible explanations for these exceptions suggest themselves:

(1) there is some compensating change in the rate of the combining reaction, or

(2) the published equilibrium curves fail to portray the equilibrium conditions at the moment of the kinetic measurements.

More extensive kinetic association data or more information about "fast" and "slow" equilibrium curves will be needed for a decision between the two.

Comparative properties of hæmoglobin and hæmocyanin.

A number of outstanding properties of hæmoglobin and hæmocyanin have been listed in Table VI. A glance at this table will show how our newer kinetic evidence has reinforced the results of equilibrium work in bringing out the essential similarity in the oxygen-binding behaviour of

TABLE VI. Comparative properties of hæmoglobin and hæmocyanin.

Properties of equilibrium curve	Hæmoglobin	Hæmocyanin
1. Shape, native state	Always sigmoid	Always sigmoid
2. Shape, purified	Probably always sigmoid	Sometimes hyperbolic: often sigmoid
3. *p*H effect, native state	None (*Urechis*) Moderate (mammals) Large (fish)	None (pure *Helix*) Moderate (*Limulus*) Large (*Maia*)
Kinetic properties		
4. Approximate time of dissociation, t_{50}, 20° C., *p*H *circa* 8·6	1/40–1/20 sec.	1/40–1/10 sec.
5. Character of dissociation	Accurately monomolecular	Monomolecular within experimental limits
6. Effect of decreasing *p*H on rate of dissociation	Increase	Increase or small change
7. Effect of increasing temperature on rate of dissociation	Great increase $Q_{10}=3·8$	Probably great increase $Q_{10}=2·2?–3·8$
8. Approximate rate of combination, k' (assuming bimolecularity)	4·0 (new)–6·3 (H.R. 1925)	2·8–3·9
Other properties		
9. Metal: Element	Iron	Copper
Pyrrol prosthetic group	Yes	No
10. Ratio, metal: oxygen molecules	1 : 1	2 : 1
11. "Met" oxidation product	Yes	Yes (Conant)
12. Addition products beside oxygen: CO	Yes	?
KCN	Probably no	Yes
13. Molecular weight	68,000 (mammals) millions (*Arenicola*)	2,000,000–5,000,000 for 2 species (no Crustacea)

these pigments, and we have seen above that the molecular weight differences need not affect this similarity. Of what significance are the other differences, such as the metallic element, the ratio of metal to oxygen bound, the existence of other loose complexes? The answer to this question is probably closely bound up with the catalytic rôle of iron and copper compounds in biological processes, and it is from the further study of oxygenation and oxidation, of oxygen transport and oxygen catalysis—perhaps by kinetic methods—that we may hope for further light on the mechanism of oxygen binding by the respiratory pigments.

Summary.

1. The reactions of oxygen with hæmocyanin take place with the same order of speed as those with hæmoglobin.

2. Under physiological conditions, the oxygen dissociation of *Limulus* serum takes about 1/10 of a second to reach half completion; that of *Maia* takes about 1/25 of a second. The association process is half completed in less than 1/300 of a second for both these pigments. These reactions are too fast to be limiting factors in metabolism.

3. The oxygen dissociation of the blood pigments has been found to be monomolecular, independently of the shape of the equilibrium dissociation curve. This is true, in particular, for sheep hæmoglobin, where the reaction has been followed throughout its entire course with a probable error of about 3 p.c.

4. Increasing the acidity of the blood pigments either greatly increases the rate of oxygen dissociation (*Maia* serum, sheep hæmoglobin, human hæmoglobin), or produces little effect on it (*Limulus* serum). It never much reduces the rate, even on the acid side of the "critical *p*H."

5. Increasing the temperature greatly accelerates the dissociation of *Limulus* hæmocyanin, as it does for sheep hæmoglobin.

6. Under identical conditions, the hæmoglobins of pig, man, sheep, and frog, have quite different rates of oxygen dissociation, as do the hæmocyanins of *Maia* and *Limulus*.

7. The equilibrium constant of dialysed *Limulus* serum as calculated from the rate of dissociation and the rate of combination with oxygen agrees with that obtained by direct measurement.

8. The bearing of these experimental results on the problem of the nature of the oxygen-binding mechanism of the blood pigments has been discussed.

I should like to express my great indebtedness to Dr F. J. W. Roughton, under whose direction this research was attempted, and to the Medical Research Council for defraying part of the expenses. The work was done in the Physiological Laboratory, Cambridge, and during a portion of the time I was aided by a grant from the Coutts Trotter Studentship of Trinity College.

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612.621

OVULATION AFTER BLOOD DILUTION AND CROSS-CIRCULATION.

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I. Introduction.

In an earlier paper [Fee and Parkes, 1929] it was shown that an amount of the ovulation-producing hormone sufficient to cause ovulation is secreted by the anterior pituitary gland of the rabbit within about 1 hour after copulation. Similar results have been obtained by Smith and White [1931] and White [1932]. Brambell and Parkes [1932 *b*] found that replacement of a considerable percentage of the blood by gum saline at about 1–1½ hours after copulation did not affect the ensuing ovulation, and therefore that an appreciable excess of the hormone over the amount actually required to produce ovulation is present at this time or can be secreted after the dilution. On the basis of experiments carried out at University College, Bangor, this excess or "safety factor" was estimated at rather over 30 p.c. of the amount in the circulation. More recent experiments, carried out in London on rabbits kept under different conditions, suggest a greater margin. Since a certain amount of the ovulation-producing hormone is presumably present in the blood of the non-mated œstrous rabbit, the existence of this large safety factor in the mated rabbit indicated that an adequate amount of hormone should be present in the cross-circulation of a mated and a non-mated rabbit to cause ovulation in both. The experiments on blood dilution, however, suggested that the lowering of the hormone concentration by distribution through two circulations might affect its availability for the ovaries. Actually, we have only succeeded in inducing ovulation in the non-mated partner if the total number of ovaries is reduced by the removal of one from each animal or of both from the mated animal.

II. Technique.

The replacement of blood by gum saline was carried out according to the technique previously described [Brambell and Parkes, 1932 *b*] and was based upon the same calculations. All experiments were carried out under urethane.

The first cross-circulation experiments were performed with the use of chlorazol blue as an anti-coagulant [see Brambell and Parkes, 1932 *a*], but later this technique was abandoned because of the toxicity of the dye, and because the use of an anti-coagulant resulted in the loss of several animals by diffuse hæmorrhage from cut surfaces. The later experiments were carried out by direct anastomosis of the vessels with Crile cannulæ, specially made for the rabbit. Circulation was established from carotid to jugular in all experiments. Direct connection, either by Crile cannulæ or by tubing in association with an anti-coagulant, has the disadvantage that it is difficult to gauge the blood flow, but the use of a reservoir in conjunction with the anti-coagulant was found to present serious difficulties owing to the small blood volume. The cross-circulation was carried out for periods varying from 2 to 4 hours, after which the animals were separated and left for a period adequate for ovulation.

III. The amount of excess ovulation-producing hormone in the mated rabbit.

The results previously recorded [Brambell and Parkes, 1932 *b*] suggested that the removal of 50 p.c. of the blood 1–1½ hours after copulation, and therefore of 50 p.c. of the hormone in the circulation at that time, inhibited ovulation. It seemed possible therefore that varying the time of removal would give some indication of the time at which the hormone is withdrawn from the circulation by the ovaries. Table I gives the results of 21 experiments carried out on these lines. Both ovaries were left in, except for Exps. 13 and 15. These results show that the surplus hormone circulating or available after copulation is even greater in these experiments than was previously estimated. The technique used was identical with that previously followed in Bangor, and the increased surplus is presumably connected with the strain of rabbit or with environmental conditions.

Attempts to inhibit ovulation by removing 60 p.c. of the blood were abandoned owing to the high mortality following removal of such a large amount, but two of three animals which survived ovulated.

TABLE I. Effect on ovulation of removing 50 p.c. of the blood at various times after copulation.

No. of exp.	Time of removal of blood after copulation hr. min.			hr. min.		Follicles ovulating
12	0	14	to	0	25	10
14	0	20	„	0	31	6
18	0	37	„	0	49	11
17	0	47	„	0	59	14
9	0	40	„	1	10	No ovulation
10	0	54	„	1	10	6
19	0	45	„	1	15	8
6	1	8	„	1	20	No ovulation
11	1	0	„	1	20	4
16	1	0	„	1	30	10
13	1	0	„	1	30	Ovulated
15	1	0	„	1	30	Ovulated
20	1	0	„	1	30	Ovulated
21	1	0	„	1	30	Ovulated
8	1	3	„	1	33	3
4	1	22	„	1	42	4
5	1	22	„	1	52	4
7	1	20	„	2	0	5
2	2	5	„	2	30	5
3	3	0	„	3	30	9
1	3	30	„	4	0	6

The anterior lobe is known to secrete within 1 hour after copulation all the hormone necessary to cause ovulation, and the above results imply that an astonishingly large excess is produced. On the other hand, it is possible that experimentally lowering the percentage of hormone in the blood results in renewed secretion by the hypophysis. Such an action, however, is unlikely in view of the results of the cross-circulation experiments. The apparent excess might also be explained if the ovaries withdrew the hormone from the circulation as fast as it was secreted during the first hour after copulation, though our results on bleeding at this time would still indicate the presence or availability of a large excess. It must be remembered, further, that the ovaries may be withdrawing the hormone from the circulation during the actual time of bleeding.

It is of importance to notice that replacement of 50 p.c. of the blood by gum saline removes not only a proportion of the hormone secreted as a result of copulation, but also some of that already present and concerned in producing the œstrous condition. Since the removal of 50 p.c. of the blood has not lowered the hormone level back to that of œstrus, it must be supposed that the post-copulation concentration is much greater than the œstrous level.

IV. Cross-circulation experiments.

The work described above suggests that it should be easy to secure ovulation in both partners of a cross-circulation between mated and unmated rabbits. Table II gives the results of nine experiments in which both partners lived for at least 15 hours after copulation of the mated partner. Most of the animals survived without difficulty and were killed after about 24 hours.

Table II. Cross-circulation of normal œstrous rabbit with mated partner.

No. of exp.	Method of cross-circulation	Ovaries removed before cross-circulation	Time cross-circulation established after copulation of mated partner (min.)	Duration of cross-circulation	Result
1	Chlorasol blue. Reservoir system	One from each animal	36	2 hr. 5 min.	4 r.f. in C.P., none in O.P.
2	,, ,,	,, ,,	45	2 hr. 5 min.	3 r.f. in C.P., 3 in O.P.
3	Chlorasol blue. Direct system	,, ,,	45	2 hr. 0 min.	3 r.f. in C.P., none in O.P.
4	,, ,,	,, ,,	55	2 hr. 3 min.	No ovulation
5	Crile cannula	None	c.c. 1 hr. 50 min. after injection of 7·5 c.c. pregnancy urine into one partner	3 hr. 0 min.	Ovulation in both ovaries of both animals
6	,,	Both from C.P., one from O.P.	95	3 hr. 0 min.	1 r.f. in O.P.
7	,,	Both from C.P.	35	3 hr. 0 min.	7 r.f. in O.P.
8	,,	None	29	3 hr. 0 min.	10 r.f. in C.P.
9	,,	None	37	3 hr. 20 min.	No ovulation

C.C. cross-circulation. C.P. copulated partner. O.P. normal œstrous partner. r.f. ruptured follicles.

These experiments show that it is possible to induce ovulation in the normal œstrous rabbit by cross-circulation with a mated partner, and thus confirm the general theory of the mechanism of ovulation in the rabbit. On the other hand, greater difficulty has been experienced in obtaining the result than would have been expected from the fact that both the concentration and absolute amount of the ovulation-producing hormone can be much reduced and yet ovulation will occur in the mated animal. It seemed probable that the surplus in the mated animal would have been enough to raise the level in the œstrous animal sufficiently to cause ovulation. In practice, however, we have failed to induce ovulation in the non-mated animal without reducing the total number of ovaries. In addition, our results show that ovulation in the unmated partner is produced most readily when both ovaries are removed from the mated animal as soon as possible after copulation. It would appear that the ovaries begin to withdraw the hormone from the circulation long before

the full amount necessary to cause ovulation has been secreted and that ovaries left in the mated partner withdraw an appreciable amount before cross-circulation can be established.

V. Summary.

1. The removal of 50 p.c. of the blood after copulation and replacement by gum saline did not prevent ovulation in a further series of rabbits.

2. This suggests an even greater "safety factor" for the ovulation-producing hormone than was previously found [Brambell and Parkes, 1932 *b*].

3. In spite of this apparent excess, ovulation was produced in the œstrous rabbit after cross-circulation with a mated partner only when the total number of ovaries in the preparation was reduced.

Our very best thanks are due to Prof. F. W. Rogers Brambell and Miss Ruth Deanesly who assisted with many of the earlier cross-circulation experiments.

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A NOTE ON THE RETINAL ACTION POTENTIAL OF THE HUMAN EYE.

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THE first to record retinal action currents from human subjects were Dewar and M'Kendrick [Dewar, 1877], who observed a deflection of their slow Thomson galvanometer in the same direction as with animals. Kahn and Löwenstein [1924] emphasized the difficulties of the method. They reproduce their best curve, obtained with the string galvanometer, but are not prepared to state that it represents the true form of the retinal response. Hartline [1925] developed the technique and showed that the method might be put to some use in comparing retinal and sensory data. The best records have been obtained by Sachs [1929]. Some of his tracings have been published by Kohlrausch [1931] and are in general agreement with our own. His success is in part attributable to long practise in the avoidance of winking and other movements of the ocular muscles.

Our original intention was to investigate the technique with a view to its possible utilization as a method of comparison with sensory events. An attempt in this direction was, however, carried out only with respect to flicker and fusion. Winks at on and off were found to cause changes of potential very similar to well-known types of retinal action current. A great deal of the time at our disposal was spent in training ourselves as subjects and in establishing the true form of the response under standard conditions. As the records gradually became consistent and repeatable within reasonable limits we decided to take up a simple problem, viz. to compare responses from the central retinal field with responses from peripheral retinal fields under optimal conditions. For such work the human eye is interesting because it is possible in the same retina to compare the action potential from an organ in which cones predominate with that from one in which rods are far more numerous than cones.

Method.

Records from the human subject were obtained with the directly coupled valve amplifier and string galvanometer which have lately been used for registering action potentials in the cat's eye [Granit, 1933; Creed and Granit, 1933]. The subject was seated opposite the stimulating apparatus with his head supported in a padded frame attached to the back of the chair. Leads were taken from the anæsthetized conjunctival sac and from the mouth by means of cotton wicks communicating with Ag-AgCl-Ringer-Locke electrodes. In the course of 20 experiments, each lasting about 30 min., some 330 records were taken.

The standard conditions ultimately adopted were as follows. The subject was in a state of considerable dark-adaptation, the lighting of the room being only such as to enable the experimenter to handle the apparatus. The stimulus was a circular sheet of ground glass illuminated from behind and of about 10 ml. brightness. It subtended about 7° at the subject's eye and carried a cross of luminous paint for central fixation. For peripheral stimulation a piece of faintly illuminated paper 30° to one side was used as a fixation mark. These conditions are optimal in that further increase of area has little effect on the amount of potential developed, increased brightness makes the suppression of winking at the beginning of the stimulus almost impossible, and light-adaptation is accompanied by smaller responses.

Results.

The normal response in central and peripheral vision.

Fig. 1 shows a response which, we are satisfied, represents a very close approximation to the true curve of retinal action potential in the human eye. The silent features of such curves are: absence of *a*-wave, small *b*-wave of about 0·2 millivolt or slightly less, small secondary rise (*c*-wave), and absence of definite off-effect. The only certain difference between central and peripheral responses is the lesser amount of potential developed in the latter. With one observer this difference averaged about 5 p.c.; with the others it was consistently about 15–20 p.c. and thus outside the limits of possible error. The latent period preceding the deflection was never less than 40σ and commonly exceeded 60σ.

It is interesting to note that the curve of potential is of the same type in the central and peripheral retina. The *b*-wave may have a more definite peak in peripheral vision, but this result was not constant and we

hesitate to accept it as significant. The use of a smaller area, restricting the number of rods participating in the central response, was found to be of little service in our attempt to compare central and peripheral effects. The responses then became too small.

Comparison with the cat's retinal action potential.

It is interesting to compare Fig. 1 with the response of the cat's eye when leads are taken from the eyeball and from the decerebration wound [see Granit, 1933] under otherwise similar conditions. The latter shows

Fig. 1. Curve of retinal action potential for observer R. G. with central vision. Diameter of stimulating disc about 5° 30′ of visual angle. The *b*-wave deflection is 0·15 millivolt. For general conditions see text. Time marker gives 100 σ and 20 σ. An interval of 1 sec. is marked in ink. Stimulus directly photographed. Observer unshielded and picking up quick oscillations which broaden the contour of the string.

a small *a*-wave, a *b*-wave with a sharp peak rising to about 0·5 millivolt, a much larger *c*-wave, and a more definite off-effect. It is difficult to believe that the human eye is so much more efficiently shunted as to account completely for these differences. A more satisfactory explanation is provided by the fact that the cat's curve of retinal action potential evoked by a stimulus one hundred times less bright resembles, both in shape and size, the human response of Fig. 1. It is therefore probable that the human eye is pitched to a higher level of stimulation and that more light is required to produce a given response. The nocturnal habits of the cat lend support to this suggestion. The human response is not significantly altered by using nose, temporal bone, or forehead instead of mouth for the indifferent electrode.

An alternative possibility, which would explain both the smallness of the *b*-wave and the long latent period in the human eye, is that the

negative component P III [Granit, 1933] of the action potential may be larger than in the cat and may hinder the deflection caused by P II. The absence of *a*-wave and of marked off-effect make this suggestion unattractive.

Flicker and retinal potentials.

Observations with intermittent light were made by Sachs [1929] on his own eye. The records from his work published by Kohlrausch [1931] are, however, all responses to continuous stimulation. The disappearance of the sensation of flicker is stated to be accompanied by the disappearance of ripples in the electrical record. We have tried to determine how closely retinal and sensory data could be correlated in this way. It was com-

Fig. 2. Retinal action potential of observer R. S. C. Intermittent light at about eight flashes a second. Standard conditions. Considerable base-line drift.

paratively easy (Fig. 2) to confirm the fact that very coarsely flickering stimuli give wavy responses. But it was found impossible, owing to the smallness of the oscillations and the unsteadiness of the base line, to determine accurately the "fusion point" in the records. An obviously flickering stimulus would give a tracing indistinguishable from one obtained with steady illumination. This does not imply that retinal and sensory "fusion" occur at different rates of alternation. Indeed it seems certain on other grounds that the retinal frequency cannot be lower than the sensory, and there is some reason for believing them to be closely related [Creed and Granit, 1933]. Our experiences described above only indicate that the question whether this relation is one of identity cannot be solved by this technique. Even with elaborate shielding of the observer and increased amplification, it is unlikely that a sufficiently steady base line could be obtained.

Discussion.

The fact that the peripheral response is similar in type to the central one, but develops less potential, is worth commenting upon. In the periphery of the retina rods are nearly twenty times as numerous as cones [Chievitz, 1889; Fincham, 1925]. It is therefore safe to assume that a much greater number of rods have been stimulated there than in the centre, even though the latter area was certainly not rod-free.

The amount of potential developed may depend upon four factors: (i) the sensitivity of the receptor (thus it increases with dark-adaptation), (ii) the number of receptors participating in the reaction (studied by Graham [1932] in an eye lacking internuncial neurones), (iii) synaptic interaction [Granit, 1933], (iv) size of negative wave. If the potential were developed in the rods and cones themselves, only the first two of these factors would come into play. The high sensitivity of rods as compared with cones in the dark-adapted eye would then presumably result in larger action potentials from the periphery than from the centre of the retina. The reverse, however, is found to be the fact. The available evidence, summarized by Granit [1933], indicates that the potential is developed in the retinal synapses or neurones, proximal to the rods and cones. Thus we are led to consider factor (iii), which at present is difficult to evaluate. According to Chievitz [1889] there are about 80 receptors for each ganglion cell 21° from the fovea, whereas in the fovea itself the ratio is 1 : 1. Far fewer bipolar and ganglion cells are therefore involved in our peripheral than in our central responses, and this may account for the difference observed. We cannot say to what extent sensitization of the rods and synaptic interaction may compensate in the periphery for a decreased number of nerve cells, but the explanation of our findings which has just been outlined is in keeping with other evidence as to the site at which retinal action potentials are developed. In view of the similarity of form between central and peripheral responses, there is unlikely to be any significant difference in the relative sizes of P II and P III.

Summary.

1. The retinal action potential of the human eye has been recorded with a string galvanometer and a directly coupled amplifier under standard conditions.

2. Responses from the periphery of the retina (30° from the fixation point) resemble those from the centre in general features, but less potential is developed. The significance of this is discussed.

3. Responses to intermittent illumination at slow rates of alternation show corresponding ripples in the electrical record. It is not possible to correlate the findings directly with sensory data on fusion frequency.

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ALBUMINURIA IN THE NORMAL MALE RAT.

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During the course of some feeding experiments an observation was made regarding the physiology of the rat to which no more than a passing reference in the literature can be found, and which, therefore, seemed worth recording. The experiments, the results of which are to be published shortly, were designed primarily to test the extent to which the growth-retarding effects of diets rich in protein could be overcome by the addition of alkali. But also, as it had been reported by Newburgh and Curtis [1928], Newburgh and Johnston [1931], Blatherwick, Medlar, Connolly and Bradshaw [1931], that kidney damage occurred if rats were given diets containing a large proportion of protein, it was decided to see whether the diets were nephropathogenic. Since, in addition to the histological evidence, these authors found increasing albumin and casts, the test animals were put into Hopkins cages in order that the urine might be tested for albumin. In the experiments in which they observed greatest damage the protein given was meat, whereas, for the sake of keeping our growth curves comparable with those of former experiments from this laboratory, casein was used.

Experimental.

The various groups of animals being studied were ingesting diets containing normal and supernormal amounts of casein, with and without additional alkali. Each group was put into a metabolism cage; the food was mixed with water into a thick paste, so that the urine might be collected with as little contamination from the food as possible. For the first few weeks of the experiment it was observed that there was no albumin in the urine collected from any of the groups. Later, however, each group exhibited albuminuria. This was the case whether the diet

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contained 20 or 70 p.c. protein, whether alkali to reduce the acidity of the urine had been added or not; it occurred alike with stock rats on stock diet as with experimental rats. In order to determine what percentage of the animals manifested this albuminuria, they were each put separately into the metabolism cages, and were each observed over a period of several days. It was noticed that about half the individuals in each group excreted albumin. When a fair number had been tested, it became apparent that whereas the urine of females was usually free from albumin, the urine of males which had reached maturity invariably contained a substance which formed a coagulum on boiling the urine and acidifying with a weak solution of acetic acid. The substance did not precipitate with acetic acid alone; the application of heat was necessary. Of a total of 70 rats examined, the 34 bucks were all excreting albumin, 33 females gave negative tests, three does showed a small amount, and of these three does, two were ingesting normal diets. There was still a positive test in the urine of males even when they had been separated from the does for several days. There being an obvious sexual factor, some of the specimens from males were examined for spermatozoa, which were present in several but not all of those investigated. We concluded, therefore, that as the source of the protein was apparently not the testes or seminal vesicles, it originated from some accessory gland such as the prostate.

The quantity present was in the vicinity of 0·5–0·8 g. per litre, as measured in an Esbach albuminimeter. No attempt was made to determine the class to which this coagulable protein belonged; it is here called "albumin" simple in the clinical sense. This constituent began to appear usually when the rats were 100–120 g. in weight, so that its incidence seemed to be connected with the occurrence of puberty. The presence of albumin in the urine is less surprising when one realizes that the rat's seminal fluid after ejection gives rise to a spontaneous coagulum known as the "vaginal plug."

An attempt to collect samples from the bladder at autopsy did not meet with enough success to justify deductions, though the few samples obtained did not give the test for albumin. In one rat, however, an anomalous structure was noticed—a spiral, gelatinous-looking structure near the neck of the bladder; and when this was teased out under the microscope, it was found to be a collection of spermatozoa, and the urine from the bladder contained sperms. We are at a loss to know what anatomical relationships in the genito-urinary system of the rat allow of the backward migration of spermatozoa into the bladder. It is possible that the relaxation of the sphincter under chloroform allows secretion to

pass back, but it does not explain the presence of the well-formed structure observed on this one occasion. In another rat, a mass, macroscopically similar, was found, but under the microscope it was amorphous. It will be realized how readily a stone can be formed in the bladder with such a foreign body as a nucleus.

From the foregoing experimental evidence, we believe that inferences as to damage of the kidney cannot reasonably be made from the detection of albumin in the urine of the mature male rat, unless it exceeds the normal amount. The quantity found by Newburgh in the urine of his experimental animals varied from 0 to 3·0 p.c. Newburgh and Curtis [1928] state that "the acidified urine from normal animals often becomes opalescent or even cloudy on being boiled," but make no distinction between urines of males and females. Longwell, Hill and Lewis [1932] observed that, in animals which were receiving excessive amounts of cystine, "there was at all times a slight albuminuria which...was no more than is normal for the male rat." In tables quoted by Donaldson [1924], the figures for nitrogen partition in the urine do not imply any difference between the urines of the two sexes, and no account is taken of protein excretion.

Summary.

A protein coagulable by heat is present in the urine of normal male rats after they have reached maturity. The amount excreted is about 0·5–0·8 g. per litre.

Grateful appreciation is herewith expressed to the Royal Society of Medicine for the scholarship which has enabled me to make these observations; to the Medical Research Council for defraying cost of materials; and to Prof. J. C. Drummond for helpful advice and criticism.

It has been brought to the notice of the author since this paper was sent for publication that similar observations have recently been published by Addis, but up to the time of going to press, no copy of the Journal containing his results has been available.

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THE DIFFUSION OF LACTATE INTO AND FROM MUSCLE.

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Certain constituents of the voluntary muscles of the frog are able to diffuse out of the muscle into a surrounding saline, and can also diffuse into the muscle if previously dissolved in the saline in a concentration sufficiently high[1]. There exists in such cases a certain concentration of the substance in saline which will be in equilibrium with the tissue. This critical concentration provides a measure of the concentration of the substance in the tissue—or rather in such part of the tissue as is concerned in the diffusion. In the case of creatine this measure of the concentration in the tissue was found by Eggleton [1930] to agree with values obtained by Dulière [1929] by direct analysis. This indicates that the partial osmotic pressure is the factor controlling the direction and extent of diffusion. Similar indirect determination of the concentration of phosphate in muscles [Stella, 1928] gave results rather lower than those obtained by direct analysis; but it must be remembered that direct estimations of the phosphate in resting muscles are liable to yield high results on account of the breakdown of phosphagen at the moment of death.

The present communication presents experiments leading to the conclusion that the concentration of lactate in both resting and fatigued muscles can be measured by the indirect "counter-diffusion" method, since the values obtained agree well with those in the literature obtained by direct analysis.

Technique.

The procedure adopted was that used by Eggleton for determining the concentration of creatine in muscle. One of a pair of resting gastrocnemii was placed in a Ringer's solution containing, for example, no lactate,

[1] Concentrations recorded in this paper were measured in mg. per 100 g. of solution; for calculation purposes these should be expressed in mg. per 100 g. water. For Ringer's solutions, however, the correction is small, and was neglected.

and the other in a solution containing, say, 60 mg. per 100 g. The amount of solution used was the same in both cases, about one and a half to two times the weight of the muscle. Gentle stirring was maintained by bubbling a suitable gas through the solution. Air and nitrogen respectively were used for experiments on resting and fatigued muscles, at a constant rate in all the cases. The tubes containing the muscle and solution were surrounded by ice in a beaker to prevent the formation of lactic acid; all the experiments were carried out at 0° C. After 2½ to 3 hours a known amount of the solution was analysed for lactate. The concentration of lactate in the solutions before diffusion was also determined. As shown by Eggleton, the equilibrium concentration of lactate in the muscle can be calculated from the formula:

$$C = \frac{L_0 l_1 - L_1 l_0}{(L_0 + l_1) - (L_1 + l_0)}, \qquad \text{.....(1)}$$

C = Equilibrium concentration in mg. per 100 g.
L_0 = Initial concentration in "high lactate" Ringer's solution.
L_1 = Final concentration in "high lactate" Ringer's solution.
l_0 = Initial concentration in "low lactate" Ringer's solution.
l_1 = Final concentration in "low lactate" Ringer's solution.

According to A. V. Hill [1930] the concentration of sodium chloride in a Ringer's solution isotonic with resting muscle is 0·71 p.c. This concentration was employed. For fatigued muscle 1·2 p.c. of sodium chloride in Ringer's solution was found to be isotonic with muscle. The concentration of potassium chloride and calcium chloride were 0·014 and 0·0125 p.c. respectively. In order to keep the *p*H of the Ringer's solution at 7·1, a buffer solution of 0·2 molar phosphate containing 65 p.c. of disodium hydrogen phosphate and 35 p.c. of sodium dihydrogen phosphate was prepared; 20 c.c. of this solution were added to 980 c.c. of Ringer's solution, giving a concentration of 12 mg. phosphorus per 100 c.c. For higher or lower concentrations of lactate, an equivalent amount of sodium chloride in the Ringer's solution was replaced by sodium lactate, so that the osmotic pressure of the Ringer's solution in high or low lactate solutions was the same.

Sodium lactate at *p*H 7·1 was prepared by myself. Lactic acid was diluted and boiled for 8 hours to convert all lactic anhydride into lactic acid. The solution was neutralized with caustic soda to *p*H 7·1, the indicator (phenol red) being used externally, lest it should have any toxic effect on the muscle cells. The diffusion was carried out at 0° C. for three hours in all cases.

Lactate in the solution was estimated by Clausen's [1922] method modified by Friedemann, Cotonio and Shaffer [1927], but without aeration [Meyerhof, 1930].

Resting muscle.

In order to obtain maximum quantities of lactate for estimation, muscles of the thigh were chosen. The frog, previously cooled to 0° C., was rendered insensible by a blow on the head or by decerebration, and sectioned immediately above the iliac bones. The abdominal muscles and the viscera in the pelvis were removed, and the skin was carefully dissected off. The legs were separated without injury to the muscles by section of the os pubis with a razor.

Table I. Concentration of lactate in resting frog muscle.

Exp.		Weight of muscles in g.		Lactate in mg. per 100 g. of Ringer's solution		Equilibrium calculated (mg. per 100 g.)
		Initial	Final	Initial	Final	
1.	*l*	10·35	10·35	0	7·2	30
	L	10·36	10·35	71·6	61·0	
2.	*l*	12·48	12·49	0	6·1	32
	L	12·49	12·50	67·7	61·0	
3.	*l*	13·95	13·96	0	5·0	23
	L	13·87	13·87	67·7	58·4	
4.	*l*	16·40	16·40	0	3·0	27
	L	16·32	16·35	71·0	65·0	
5.	*l*	16·50	16·51	0	7·7	26
	L	15·36	15·35	79·2	63·4	
6.	*l*	15·97	16·02	0	7·2	29
	L	15·94	15·97	37·7	36·0	
7.	*l*	4·71	4·65	0	6·4	24
	L	4·50	4·57	37·7	31·1	
8.	*l*	4·35	4·35	0	9·9	17
	L	4·35	4·35	37·7	26·4	
9.	*l*	1·36	1·37	0	6·2	24
	L	1·39	1·34	44·9	39·7	
10.	*l*	1·83	1·83	0	3·0	13
	L	1·84	1·85	37·0	31·5	
11.	*l*	1·22	1·23	0	4·0	21
	L	1·23	1·24	37·0	34·0	
12.	*l*	1·45	1·45	0	5·3	18
	L	1·25	1·24	37·0	31·6	
13.	*l*	0·98	0·98	0	4·5	16
	L	0·99	0·98	39·4	33·3	
14.	*l*	2·60	2·60	0	3·0	23
	L	2·65	2·64	46·0	43·0	
15.	*l*	2·84	2·83	0	2·5	14
	L	2·90	2·89	47·2	41·1	

l, low lactate Ringer's solution; *L*, high lactate Ringer's solution. Exps. 1–6 were performed on thigh preparations. Exps. 7–15 were performed on gastrocnemii, or calf preparations.

Consistent results of 30 mg. per 100 g. of solution were obtained for the concentration of lactate in equilibrium with frog muscle. To confirm this, muscles were placed in a modified Ringer-lactate solution containing 30 mg. lactate per 100 g. of solution, and it was found that the muscles were in equilibrium with regard to lactate; the concentration of lactate in solution remained constant. This figure, 30 mg. per 100 g., is twice the amount of the accepted figure for resting frog muscle. This was thought to be probably due to partial asphyxia of the muscles, or due to the dissection of the recti muscles which are attached to the skin; even though the injured fibres were cleared away there might be fibrillar contractions taking place, giving rise to lactic acid in the muscle. Therefore they were abandoned and gastrocnemii, preferably with their bony attachments, were selected; in other cases the second segment of the leg with the muscles of the calf were taken. Both these preparations can be made without any injury to the muscles concerned. The diffusion technique was unaltered. The preparations were reweighed at the end of each experiment and found to have neither gained nor lost in weight; therefore the solution was isotonic. For equation (1) to be valid, there should be no transference of water into or from the muscle. The muscles were stimulated and found to be excitable. From each tube 2 g. of the solution were taken and analysed for lactate.

An average of the results recorded for Exps. 7–15 shows a concentration of 20 mg. per 100 g. of solution. From this figure, it is easy to arrive at the concentration of lactate in the muscle. Since the muscle contains 80 p.c. by weight of water the concentration of lactate in the muscle is 16 mg. per 100 g. muscle.

Fatigued Muscle.

In this case the gastrocnemius muscles of the frog were fatigued by causing them to contract isometrically. They were dissected out, weighed and introduced into the tubes containing modified Ringer-lactate solutions. In order to prevent oxidative recovery the solution was covered in the earlier experiments with a layer of liquid paraffin. The steady gentle stirring was maintained by bubbling nitrogen through the solution. The use of liquid paraffin renders it impossible to find if the weight of the muscle had remained constant during the experiment; but the muscles could be tested to see if they were still excitable. Later on the use of liquid paraffin was given up and the tubes were stoppered, after the air had been displaced by nitrogen. The weight of the muscles in these cases

was noted and found to be constant. At the conclusion of the experiments the muscles were found to be still excitable.

TABLE II. Concentration of lactate in fatigued frog muscle.

Exp.		Weight of muscles in g. Initial	Final	Lactate in mg. per 100 g. of Ringer's solution Initial	Final	Equilibrium calculated (mg. per 100 g.)
1.	*l*	—	—	0	22	232
	L	—	—	512	487	
2.	*l*	—	—	0	25	220
	L	—	—	500	470	
3.	*l*	—	—	0	23	230
	L	—	—	502	476	
4.	*l*	1·24	1·25	0	16	270
	L	1·25	1·25	502	489	
5.	*l*	1·62	1·62	0	14	214
	L	1·64	1·63	412	399	
6.	*l*	1·36	1·39	0	24	218
	L	1·37	1·36	412	391	
7.	*l*	0·92	0·96	11	34	214
	L	0·90	0·90	418	394	
8.	*l*	1·43	1·44	11	30	205
	L	1·50	1·49	418	396	
9.	*l*	1·09	1·10	11	36	286
	L	1·09	1·09	418	406	

The average equilibrium concentration is 238 mg. per 100 g. of solution. This corresponds to a concentration of 190 mg. of lactate per 100 g. of fatigued frog muscle.

SUMMARY.

Studies of diffusion of lactate into and out of resting and fatigued frog muscles indicate an apparent concentration of lactate in the water of the muscle of 20 and 238 mg. per 100 g. respectively. This is in good agreement with the values obtained from direct analyses of such muscles by other workers. It follows that all the water of the muscle is available to dissolve lactate.

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AN ISOLATED MAMMALIAN HEART PREPARATION CAPABLE OF PERFORMING WORK FOR PROLONGED PERIODS.

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In experiments performed to determine how far the lungs influence the increased diastolic filling of the heart as a result of a reduction in respiratory (intrathoracic) pressure, it was found necessary to replace the lungs of a heart-lung preparation by a blood oxygenation apparatus [Daly, 1927]; for the oxygenator a modified form of the apparatus designed by Hooker [1915] and Drinker [1922] was used. In such an isolated heart preparation performing work, it was found that the heart quickly became hypodynamic in spite of good oxygenation of the blood, the average duration of five experiments being only 36 min. (limits 25–60 min.). This rapid onset of cardiac failure, as compared with the heart-lung preparation which lasts at least five hours if not overworked, was also observed independently by Verney [1927], and has since been observed by others. At that time no attempt was made to investigate the cause of the heart failure in the isolated heart preparation, but recently we have carried out a number of experiments which have enabled us to maintain the isolated heart in good condition for a period of time at least equal to that generally obtained with the heart-lung preparation and also have disclosed conditions which may be of interest relative to all perfusion experiments performed with defibrinated blood.

We do not propose to describe in detail the various forms of apparatus used in some twenty-four experiments before final success was obtained, but will merely mention briefly the cause of our early failures and particularize those experiments which are relevant to the points we wish to discuss.

Methods.

In all our experiments a Starling's heart-lung preparation was made to run on defibrinated blood and then converted to an isolated heart preparation performing work by replacing the lungs with some type of

Hooker-Drinker blood oxygenating apparatus. In order to do this a cannula was inserted into the central end of the pulmonary artery or into its left branch and the blood conducted to the oxygenator and finally back

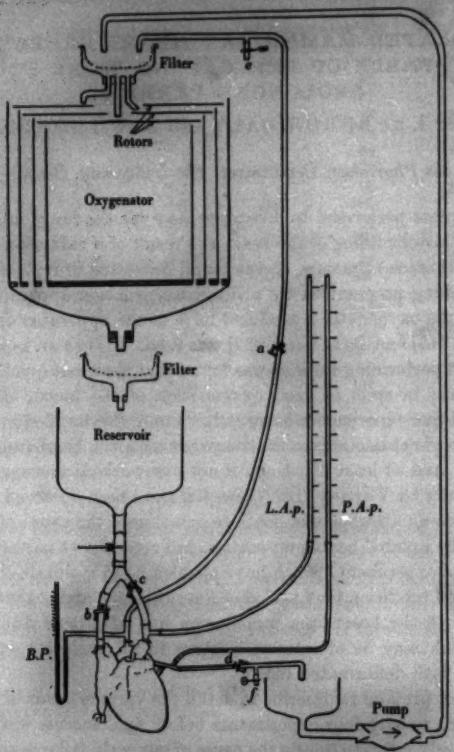

Fig. 1. General arrangement of apparatus.

to the left auricle through a cannula tied into the left auricular appendix. The lung roots were then tied off and the lungs removed. Owing to the height of the oxygenating apparatus it was necessary to insert a pump to lift the blood from the level of the heart to the top of the apparatus, thus saving the right heart from having to put out blood at a pressure above

the normal. The position of the oxygenator relative to the heart was not always the same in every experiment, but, whatever the arrangement, the oxygenation of the blood from the right heart, at a normal or less than normal pulmonary arterial pressure, was always achieved. It is convenient at this stage to describe in detail the method finally adopted and later to indicate the necessity for taking certain precautions to ensure success.

In all experiments dogs were used, anæsthetized with 0·1 g. per kg. body weight of chloralose injected intravenously after induction with chloroform and ether, except in the last two experiments in which the heart-lung preparation animal and the dogs from which blood was collected were anæsthetized with 0·04 g. per kg. body weight of "Nembutal" injected intravenously under local anæsthesia without induction with chloroform and ether. The blood collected for the experiment is defibrinated and filtered through a tower of four or five cambric filters and then kept at 37–39° C. until ready to be used; it is filtered again just before use. The cambric filters had a mesh, measured dry, of approximately $50 \times 100\,\mu$. A Starling's heart-lung preparation is made and arranged so that the left ventricle pumps the peripheral systemic blood to a cambric filter placed above the oxygenator (Fig. 1). The blood then drains through the oxygenator on to a second filter thence to a reservoir which has a capacity of 1·5 litres and is placed in a thermostat. A screw clip (*a*) on the rubber tubing is used for adjusting the systemic pressure in the region of 110 mm. Hg. A cannula with a side tube for taking the left auricular pressure is inserted into the left auricular appendix and connected by a Y-piece to the reservoir outflow tube, a screw clip (*c*) being placed tightly on the rubber connection. By this arrangement the blood from the reservoir may be diverted either to the right or to the left auricle. Pure oxygen is then passed through the oxygenator and the rotors set in motion, the screw clip (*c*) being open and screw clip (*b*) closed so that the blood from the reservoir flows into the left auricle and ventricle. Thus, the peripheral systemic blood is oxygenated by the oxygenator and only the coronary blood by the lungs before it mixes with the reservoir blood in the left auricle. Positive pressure artificial respiration is carried out with the Starling Ideal respiration pump. This concludes the first stage of the operation.

In the second stage, a cannula with a side tube is inserted centrally into the left pulmonary artery, both lung roots are ligated and the lungs removed; the coronary blood which passes out from the left pulmonary arterial cannula is collected in the reservoir of a Dale and Schuster [1928] pump regulated to the coronary flow, and conveyed to

the filter above the oxygenator. The side tube of the pulmonary cannula is connected to a manometer so that the pulmonary arterial pressure may be taken, and a screw clip (*d*) enables this pressure to be maintained at any

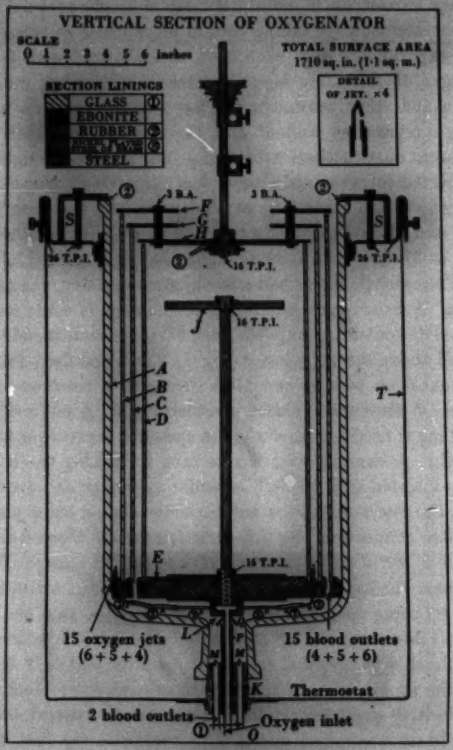

Fig. 2. Oxygenator.

desired level. By adjustment of the screw clips *b* and *c* the total quantity and the proportion of blood dealt with by each side of the heart can be adjusted at will. Since the blood-pressure of each side of the heart can also be adjusted separately, the quantity and type of work performed by each

side of the heart can be varied as desired. The blood flows are measured through the side tubes *e* and *f*, but in a few experiments we recorded blood flows with Pitot tubes. When both screw clips *b* and *c* are open, the blood flow from the left pulmonary artery consists of the amount flowing from the reservoir into the right auricle plus the coronary flow. In order to measure the coronary flow under these conditions it will be necessary to measure the flow from the side tube *f* and subtract from it the amount coming from the reservoir into the right auricle obtained by insertion of a flow recorder at *b*. For the purpose of comparing one experiment with another the peripheral systemic flow and resistance are kept approximately constant and the left auricular pressure is taken as the criterion of the functional capacity of the heart. Except for a small slit to allow of insertion of the left auricular cannula, the pericardium is kept intact.

Our early experiments had indicated the need for not only a larger oxygenator but one which would be able to deal with sudden changes in flow. Since existing forms of oxygenators at that time were either too small or unable to deal satisfactorily with a fluctuating flow it was decided to design an oxygenator sufficiently large to deal with flows much greater than we anticipated for the present experiments. Fig. 2 is a scale drawing of this apparatus which is in effect three Hooker-Drinker oxygenators arranged concentrically. The blood is distributed by three jets on to the ebonite discs *F*, *G*, *H* and spun out on to the walls of the glass cylinders *A*, *B*, *C* respectively. The films of blood flowing down these cylinders are oxygenated by oxygen from 15 jets (6 for *A*, 5 for *B* and 4 for *C*) mounted in the ebonite base *E*. A similar number of holes in this base serve as blood outlets to the bottom of the bell jar *A* and hence to the blood reservoir by the two glass tubes *M*, *M'*. The cylinder *D* is to prevent waste of oxygen in the centre of the apparatus. The oxygen passes into the apparatus through the tube *O* to the jets by way of the distributor *L* (Fig. 3). The tube *O* slips into the tube *P* mounted in the rubber bungs *K*. The base *E* and the three cylinders *B*, *C*, *D* can thus be removed from the bell jar *A* by the handle *J* (Fig. 4). *Q* is a rubber baffle to prevent any blood spray escaping between *O* and *P*.

The apparatus is heated by immersing the jar *A* in water at 38° C. in the galvanized tank *T*, the jar being held down by the clamps *S*. The water is heated by three 500-watt electric immersion heaters controlled by resistances, and stirred with two propellers not shown. The dimensions of the apparatus can be seen from the scale drawing and it should suffice to mention that the bell jar *A* is 18½ in. high and 11¼ in. inside diameter.

The inner surfaces of *A*, *B*, and *C* are respectively 650, 570 and 490 sq. in., *i.e.* a total surface area of 1710 sq. in. (1·1 sq. m.). The apparatus is capable of dealing with relatively large volumes of blood. While not intended to cope with flows much over 600 c.c. per min. a sudden gush of blood of even as much as 2500 c.c. per min. could be accommodated without overflowing. The volume of blood pooled in the apparatus at a

Fig. 3.

Fig. 4.

Fig. 3. Distributor of oxygenator.

Fig. 4. Inner cylinders of oxygenator.

flow of 660 c.c. per min. is 205 c.c. using cylinders *A* and *B* only, 300 c.c. using all three cylinders and 350 c.c. at 850 c.c. per min. All metal parts of the apparatus are heavily nickel plated. The apparatus was *completely* dismantled and cleaned after each experiment with soap and water and rinsed with plenty of clean water, and before each experiment the filming surfaces were treated with cotton wool and spirit.

Results.

Preliminary experiments.

The technique of our earlier experiments was essentially the same as described except that the defibrinated blood was first filtered through muslin and then through one cambric filter of 100 × 200 μ meah when measured dry, and no filters were inserted above the oxygenator reservoir. Our results confirmed those of Daly and Verney in that cardiac failure associated with a rise in left auricular pressure occurred early on in the

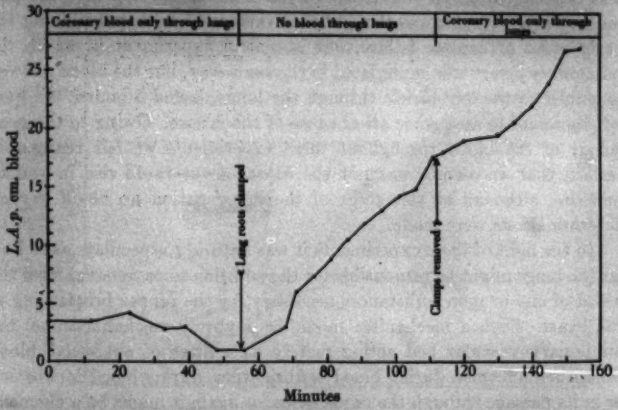

Fig. 5. Effect on left auricular pressure of changing from a heart-lung preparation, in which only the coronary blood flows through the lungs, to an isolated heart preparation. Coarse cambric filters used.

experiment. In searching for a cause of this failure we tried stopping the operation at the end of the first stage so that only the coronary blood flowed through the lungs, the peripheral systemic blood passing through the oxygenator. It was then found that the left auricular pressure remained nearly constant for at least one hour and a quarter, this being the longest period tried, but on performing the second stage of the operation and switching over to the isolated heart preparation, the venous pressure started to rise rapidly. In this series of experiments instead of ligating and removing the lungs for the second stage of the operation we used gastro-enterostomy clamps guarded with rubber tubing for cutting off the pulmonary circulation. This procedure had the

advantage of enabling us to return to the conditions of the first stage by removing the clamps and clipping the rubber tubing on the pulmonary arterial cannula. Fig. 5 shows the effect of running the preparation with only the coronary blood going through the lungs and then changing over to the isolated heart preparation and finally back again to the initial conditions. It is clear that changing over to the isolated heart preparation rapidly diminished the functional capacity of the heart as evidenced by the rise in venous pressure, moreover a return to the initial conditions did not restore the efficiency of the heart. In these experiments the blood was collected from the right heart by a cannula inserted into the pulmonary artery, and it was thought that this might be responsible for the early onset of cardiac failure, but a control experiment in which the pulmonary artery was cannulated in the same way, but the blood allowed to return to the left auricle through the lungs, lasted 5 hours. We were left therefore to seek some other cause of the failure. Owing to the good colour of the blood throughout these experiments we felt reasonably certain that anoxæmia was not the cause of the rapid rise in venous pressure, although at this stage of the investigation no blood oxygen determinations were made.

In the light of these experiments it was natural to speculate as to how far the lungs might be responsible for the addition to or removal from the blood of one or more substances necessary for the proper functioning of the heart. Such a mechanism might be a purely mechanical one, the pulmonary vascular bed acting merely as a filter of abnormal blood constituents formed during blood defibrination, during blood incubation or in its passage through the oxygenator, or again it might be a chemical one, and we were at once attracted to the hypothesis that histamine addition to or removal from the blood by the lungs might play some part. We had in mind the investigations of Best, Dale, Dudley and Thorpe [1927] which demonstrated the presence of large quantities of histamine in the lungs, the significance of which is as yet unknown. Our results, however, gave no support to the view that lack of histamine is responsible for the hypodynamic condition of the heart. In one experiment 0·05 mg. histamine per minute was added to the blood of the isolated hypodynamic heart preparation for 14 min., and in a second experiment 0·06 mg. per minute for 23 min., without any apparent effect on the heart. The method of adding the histamine to the blood by continuous infusion renders it difficult to compare our results with those of other workers. In experiments on the heart-lung preparation, Führer and Starling [1913] obtained marked dilatation of the heart succeeding a transient volume

diminution, with a dose of 0·5 mg. histamine injected into the pulmonary artery; Rühl [1929] and Rössler [1930] obtained similar results with larger doses (0·5–2·0 mg.); Müller, Salomon and Zuelzer [1932] found no evidence of cardiac damage by the addition of single doses of less than 0·1 mg. histamine. Larger doses, 0·5 mg., damaged the fresh heart but not the heart which had previously been treated with successive small doses of histamine.

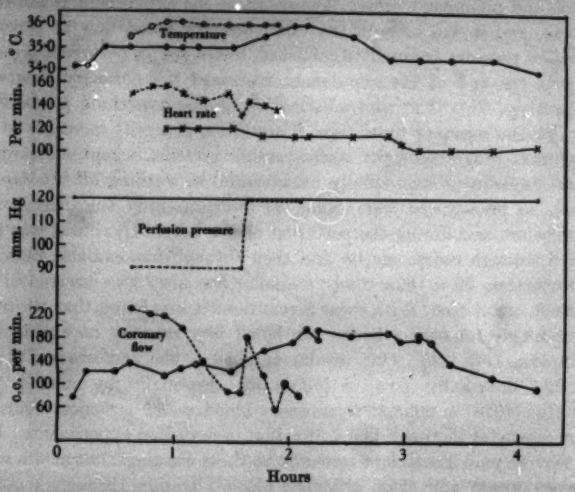

Fig. 6. Isolated perfused heart. (*a*) The broken lines indicate the events taking place when the blood is filtered through coarse cambric. (*b*) The continuous lines, the events when the blood is filtered through fine cambric. In (*a*) the coronary blood flow shows a rapid and progressive diminution.

The histamine content of the hearts after perfusion or after performing work as a heart-lung preparation or heart-oxygenator preparation showed no significant departure from the values obtained from normal hearts.

Isolated perfused heart.

Attention was then directed to the question of blood filtration. Up to this time the blood had been filtered immediately after defibrination through one layer of muslin and a layer of cambric having a mesh of $200 \times 100\,\mu$ when measured dry. In general it was filtered again through

cambric just before use. Using this method of blood filtration we set up an isolated heart preparation perfused through the aorta with a Dale and Schuster pump. Cannulæ were placed in the left ventricle through the left auricle, in the central end of the pulmonary artery and in the right auricle to enable the coronary blood and any blood finding its way past the aortic valves to have a free exit. An oxygenator was used for oxygenating the blood. The coronary blood was collected from the right auricle and pulmonary artery. The result of one experiment is shown in Fig. 6*a*, and it will be seen that the coronary blood flow at constant perfusion pressure gradually diminished, and a rise in perfusion pressure towards the end of the experiment increased the coronary flow only temporarily. This is in contradistinction to the heart-lung preparation in which the coronary blood flow, if anything, generally increases as the experiment progresses if the mean systemic pressure is kept constant. A second experiment was equally unsuccessful in warding off the cardiac failure, so precautions were taken to filter carefully the defibrinated blood before and during the perfusion of the heart. The blood was first filtered through coarse muslin and then through five cambric filters of approximately $50 \times 100\mu$ mesh; a similar fine filter was inserted in the perfusion circulation. With these precautions it was found that the heart beat strongly for over 4 hours, the blood flow showing no evidence of diminution (Fig. 6*b*). Our results emphasize the importance of the warnings issued by Brodie [1903] and especially by Anrep and Häusler [1928] in relation to coronary blood vessel perfusion regarding the necessity of thorough blood filtration in perfusion experiments. It is felt that we paid insufficient attention to these warnings, but at the same time we would add that, although blood filtration through a coarse muslin filter followed by passage through a $100 \times 200\mu$ mesh cambric is sufficient to enable the heart-lung preparation to perform work for some hours, such filtration is not sufficient for the isolated heart preparation.

Isolated heart performing work.

Having recognized the importance of good blood filtration we returned to the experiments on the isolated heart preparation performing work. The apparatus already described in detail and the $50 \times 100\mu$ cambric filters were used. The first two experiments in which operations apparently went without a hitch lasted 3 and $1\frac{1}{2}$ hours after switching over to the isolated heart from the heart-lung preparation. In spite of the fact that one of the preparations lasted 3 hours we were not satisfied with this result. A third (Fig. 7) and fourth experiment, however, lasted 6 and $6\frac{1}{2}$ hours from the

start of the heart-lung preparation, the heart isolated from the lungs performing work for 5 and 4½ hours respectively; the latter experiment was terminated while the heart was still in good condition. There were slight differences in the technique in these four experiments, which should be mentioned because the third and fourth preparations were in a far better physiological condition than the first and second. Chloralose injected intravenously preceded by chloroform and ether induction was

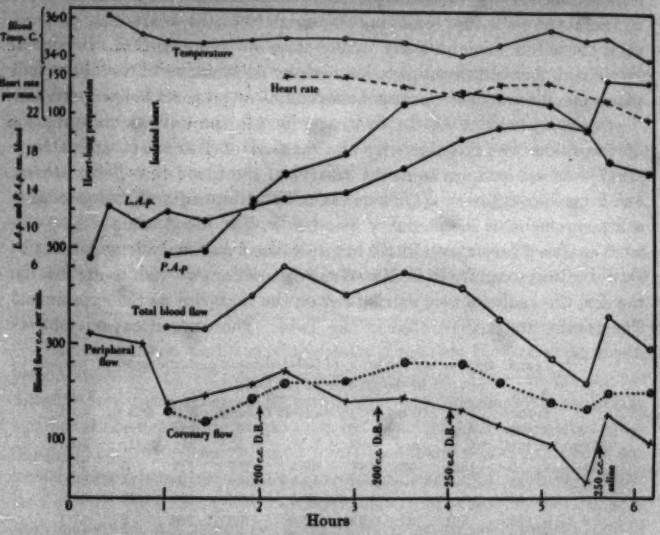

Fig. 7. Dog. 13·0 kg. Isolated heart-oxygenator preparation. Pericardium closed. *B.P.* = 107–111 mm. Hg. *L.A.p.* = left auricular pressure. *P.A.p.* = pulmonary arterial pressure. *D.B.* = defibrinated blood.

given to the preparation animals of the first and second experiments, and the extra blood was collected from animals under chloroform and ether anæsthesia. Nembutal injected intravenously under local anæsthesia was administered to all the animals used for the third and fourth experiments in the hope that the absence of chloroform during induction would improve the condition of the heart preparation. In the first three experiments defibrinated blood from the thermostat was added from time to time to the reservoir to make up any volume loss due to water evaporation

or to slight leakages, and in these experiments it was noticed that the cambric filters became clogged with a gelatinous blood film and had to be changed from three to five times in the course of the experiment. The film sometimes contained small lumps of what at first sight appeared to be fine blood clots, but on examination were found to be friable aggregations of material somewhat similar to that forming the film; that the concentrated nature of the blood plays some part in the formation of this film is not improbable. In the fourth experiment we added all the available blood to the reservoir before removing the lungs from the heart-lung preparation, *i.e.* before beginning the second stage of the operation. It may be significant that in this preparation little or no blocking of the filters took place, one filter in each position being sufficient for the whole experiment.

Oxygen consumption. In two experiments the oxygen consumption of the heart was measured by the method of Barcroft and Dixon [1907]. Blood samples from the reservoir, the blood first being stirred, and from the outflow tube (*f*) were taken either immediately before or after a measurement of the coronary blood flow. The blood samples (2·0 c.c.) were analysed for oxygen in Haldane's blood gas analysis apparatus by Dr J. L. Berry and Dr R. G. MacGregor to whom we wish to express our thanks; the analyses were carried out on the same day as the experiment. The results are incorporated in the Table. The oxygen capacity of the

TABLE. Oxygen consumption of isolated heart performing work in the absence of the lungs.

Exp.	Time from start hr.	min.	Dog wt. kg.	Heart wt. g. dry	wet	Output L./hour L.V.	R.V.	B.P. mm. Hg	P.A.p. mm. Hg	Total work kg.m./ hour
1		5	13·0	33	187	20·3	9·7	111	5	30·0
	4	32	—	—	—	13·1	10·1	108	15	20·5
	5	14	—	—	—	20·2	12·2	107	12	30·0
2		18	6·0	21	93	15·8	3·6	134	2	27·7
	4	18	—	—	—	7·6	4·0	130	6	13·1

The figures given below are obtained from the same two experiments at corresponding time intervals.

Exp.	Time from start hr.	min.	Reservoir blood O_2 c.c. p.c. Capacity	Content	Coronary blood O_2 c.c. p.c. Capacity	Content	Coronary flow c.c./ min.	O_2 consumption c.c. Per hour	Per g. heart wet wt./ hour	Per g. ht./kg.m./ hour Wet wt.	Dry wt.
1		5	19·1	16·3	19·3	13·6	162	262	1·4	0·05	0·26
	4	32	30·2	24·8	31·9	21·3	168	353	1·9	0·09	0·52
	5	14	27·1	23·8	26·5	15·3	204	1040	5·6	0·18	1·05
2		18	18·5	18·35	18·5	8·0	60	373	4·0	0·15	0·64
	4	18	27·0	25·5	27·0	17·0	66	337	3·6	0·28	1·22

P.A.p. = pulmonary arterial pressure. L.V. = left ventricle. R.V. = right ventricle.

second arterial and venous blood sample in each experiment shows a large increase which is due to blood concentration consequent upon the evaporation of water from the blood on the large surfaces of the oxygenator cylinders. In the first experiment a colorimetric determination of the hæmoglobin percentage of a blood sample taken 4 hours 32 min. from the start gave a value of 160. The total amount of circulating blood at this time was 1100 c.c. and the amount at the beginning of the experiment 1850 c.c., the quantity lost therefore being 750 c.c. in 4·5 hours or 167 c.c. per hour. From these figures the calculated hæmoglobin percentage is 168, which is in fair agreement with the value obtained from the colorimetric determination. Such a rapid rate of water loss from the blood is a serious disadvantage of the oxygenator, and no doubt a closed system apparatus similar to that designed by Bayliss, Fee and Ogden [1928] would overcome this difficulty. Early on in the investigation we attempted to use two of their oxygenators in parallel but were not fortunate enough to get them working properly without "spating" at varying blood flows, although at constant blood flows we found them satisfactory. Nine minutes after the second reading of the first experiment (Table) 250 c.c. of normal saline were added to the reservoir blood, and this probably accounts for the lowered oxygen capacity of the blood at 5 hours 14 min.

The oxygen consumption of the isolated heart in the two experiments varied from 1·4 to 5·6 c.c. oxygen/g. heart/hour. These values do not differ widely from those of Evans [1912] who obtained corresponding figures for the heart-lung preparation of 3·5–6·0 and for the isolated lungs of approximately 0·9 c.c./g. heart/hour; by subtraction the intake of the isolated heart being 2·6 to 5·1 c.c. oxygen/g. heart/hour. Details of these two experiments are given in the Table, from which it will be seen that the oxygen utilization per kg.m. of work increases towards the end of an experiment of approximately 4 hours. In view of the fact that we have only undertaken the measurement in two experiments and did not adopt a method of integration, we do not wish to stress this point.

In the perfused heart Barcroft and Dixon [1907] give values from 0·6 to 5·0 c.c. oxygen/g. heart/hour, McGinty and Miller [1933] 2·4 to 5·7 and Eichholtz and Hilton [1925] 2·2 to 6·0. In the heart-lung preparation Evans and Starling [1913], using two different methods, give figures varying from 1·1 to 4·3. In the de Barenne [1919, 1921] modification of the heart-lung preparation Satani [1931] found an intake of 2·0 to 9·8 and Yoshioka [1932] of 1·6 to 6·2. In this connection Evans [1914] and Evans and Matsuoka [1915] have shown that the oxygen intake of the isolated heart is not only determined by the quantity but also by the quality of work performed.

In the isolated perfused heart the organ is doing little or no external work, and in the de Barenne preparation only the left side of the heart performs work. In our own prepara-

tion, as we have used it, the left side of the heart is delivering blood at the normal blood-pressure, whereas the right side only puts out the coronary blood flow at less than the normal pulmonary arterial pressure; finally, in the Starling heart-lung preparation and the heart *in situ* both sides of the heart carry out the normal amount of work.

In order to be able to compare the oxygen consumption of different heart preparations we have calculated the oxygen consumption in c.c./g. heart dry wt./kg.m./hour. At the end of a long experiment the heart capillaries are congested with blood and the muscle may be œdematous with a consequent increase in its weight, and this may be a source of error when comparing the weights of different hearts. To obtain the dry weight the aorta and pulmonary artery are removed close to their origin from the ventricles and the heart cavities are freed from blood by washing after opening all chambers of the heart. The organ is freed from water by draining and weighed, giving the wet weight, and then minced and dried to constant weight at 100° C. In two normal animals the ratio of dry to wet weight of the hearts was 1 : 3·5 and 1 : 4·0, and the hearts of Exps. 1 and 2 (Table I) 1 : 5·7 and 1 : 4·4 respectively. The external work of the heart was calculated by the formula of Evans [1918] although the velocity factor is hardly significant in our experiments.

The coronary blood flow. When the screw clip *b* is closed and the reservoir blood feeds the heart through the left auricle only, the total coronary blood is measured from the side tube *f*. Anrep [1926] considers 40–60 c.c./g. heart/hour to represent the average magnitude of the coronary circulation at a blood-pressure of 100–110 mm. Hg. The blood-pressure in some of our experiments has been somewhat higher than this, but the coronary flow has been within or only just outside the limits given by Anrep.

In two experiments we determined the influence of the addition of "fresh" defibrinated blood to the reservoir. By "fresh" defibrinated we mean blood which had been incubated at 37–39° C. for 3–7 hours and had not circulated through the heart-lung preparation: Eichholtz and Hilton [1925] used the term in this sense. No obvious permanent effect on the coronary circulation was observed (Fig. 7), although a transient one might not have been revealed by our methods of measurement. This confirms the findings of Eichholtz and Hilton on the heart-lung preparation; these workers replaced nearly all the reservoir blood with "fresh" defibrinated blood, whereas we added 200–250 c.c. of "fresh" blood to 500–1000 c.c. of circulating blood so that the "fresh" blood concentration in our experiments was smaller than in theirs.

Heart volume. In the heart preparations which lasted for longer than 4 hours it was surprising to find that the heart showed no dilatation

except quite at the end of the experiment. In the two experiments given in the Table, the pericardium remained intact except for a slit to allow of the insertion of the left auricular cannula, yet after 4 hours in each experiment the pericardium appeared to be quite slack and the heart small and hard. In view of the loss of efficiency at this stage as compared with the beginning of the experiment it was expected that the heart would be dilated. Since pure oxygen was passed through the oxygenator the small size of the heart was probably due to the acapnic condition of the blood [Jerusalem and Starling, 1910]. Insulin, which abolishes the progressive dilatation of the heart in the heart-lung preparation [Müller and Visscher, 1927], and glucose which has the same effect [Bayliss, Müller and Starling, 1928] were not added to the blood. At first sight it would appear that the increased oxygen consumption of the heart (Table) without an increase in diastolic volume is against the findings of Starling and Visscher [1927] and Fee and Hemingway [1927]. But Starling and Visscher expressly state that the oxygen consumption of the isolated heart (*i.e.* heart-lung preparation) maintained under constant chemical and temperature conditions is determined by its diastolic volume. They were able to maintain constant the chemical conditions during the relatively short period of time taken by their observations, whereas in our long experiments, the chemical condition of the blood at the end of the experiment probably differs markedly from that at the beginning. The absence of the lungs may possibly play some part in the phenomenon.

Discussion.

During the investigation we had to perform a number of control experiments in order to eliminate various possible causes of early cardiac failure. These experiments showed that the following factors produced no detectable alteration in the performance of the isolated heart beating on well-filtered blood over a period of 5 hours as compared with that of the heart-lung preparation: (*a*) the removal of any nervous control arising in the lungs, (*b*) the absence of substances formed by the lungs and necessary for the normal contractile processes of the heart, and (*c*) loss of water from the blood due to evaporation in the oxygenator [Daly and Thorpe, 1933].

Our experiments, however, indicated the necessity for good blood filtration and we attach importance to two main findings, the first, that an isolated heart preparation, performing work and running on "fresh" defibrinated blood added to the reservoir after the preparation is made,

has not lasted longer than 1 hour unless the blood was well filtered before and during the experiment, and the second, that in all such successful preparations the cambric filters became clogged with a gelatinous film. We find, therefore, that fine cambric filters are as effective as the lungs in preventing the early onset of cardiac failure in heart preparations performing work and running on "fresh" defibrinated blood. To account for this phenomenon two hypotheses are put forward. The first, that the cambric filters in the heart-oxygenator preparation and the lungs in the heart-lung preparation are acting as simple mechanical filters of normal or abnormal blood constituents, which if allowed to enter the circulation gradually lead to occlusion of the coronary blood vessels. By abnormal constituents we mean those formed by changes in the defibrinated blood giving rise to a gelatinous material containing very small clots, fine shreds of fibrin or masses of blood corpuscles clumped together. The second hypothesis assumes the presence in the blood of substances responsible for the early onset of cardiac failure which are formed when the blood is defibrinated or when in the thermostat or in the artificial circulation, these being removed by the lungs in the heart-lung preparation and by the cambric filters, *e.g.* by adsorption, in the heart-oxygenator preparation. In this connection, the work of Zipf [1931] and others has demonstrated such a possibility. Our experiments do not enable us to decide whether one or both of these hypotheses are correct; both appear to satisfy the experimental findings. It may be that the postulated blood constituents or the substances of the second hypothesis are only formed in the oxygenator. Nevertheless, the lungs seem able to deal with such substances since in a heart-lung-oxygenator preparation without special filtration there was no early onset of cardiac failure.

The premiss that the lungs or cambric filters may remove substances from the blood which are responsible for the appearance of early cardiac failure leads to a speculation as to their nature, and attention was naturally attracted to the possibility that the vaso-tonins were responsible. It has been shown by Eichholtz and Verney [1924], Hemingway [1931] and Bayliss and Ogden [1933] that these substances cause constriction of the vascular system in the perfused kidney. Eichholtz and Hilton [1925], however, could obtain no evidence that "fresh" defibrinated blood had any effect on the coronary flow in the heart-lung preparation; in their experiments the "fresh" blood had to pass once through the lungs before reaching the coronary vessels and there is the possibility that the vaso-tonins might have been destroyed even in this short time [see Eichholtz and Verney, 1924]. As mentioned previously

our own observations confirm those of Eichholtz and Hilton in the heart-oxygenator preparation with blood filtered through fine cambric. It would appear, therefore, that if the early onset of cardiac failure in the heart-oxygenator preparation running on poorly filtered blood is due to the presence of vaso-tonins, they are removed by the insertion of fine cambric filters. For perfusion of the coronary vessels, Anrep and Häusler [1928] found it necessary to circulate the blood in the heart-lung preparation for about half an hour, otherwise the defibrinated blood caused vigorous and protracted vaso-constriction. In all our experiments, except one, only about half the total blood finally used circulated through the heart-lung preparation before switching over to the isolated heart circuit, the other half was then added to the reservoir. Under these conditions we observed no significant coronary vaso-constriction (Fig. 7) provided fine cambric filters were used. In the excepted experiment all the blood eventually used for the heart-oxygenator preparation was circulated through the heart-lung preparation and in this case little or no clogging of the filters occurred, but we hesitate to lay stress upon this observation in a single experiment. It is not improbable that the formation of the gelatinous film on the filters may in part be determined by the biological properties of the bloods obtained from the two or three animals used and their effect upon one another when mixed together. It may be said that our observations taken in conjunction with those of Anrep and Häusler suggest that the insertion of cambric filters in the isolated heart preparation, to which "fresh" defibrinated blood is added from time to time, obviates the necessity of circulating the "fresh" blood through a heart-lung preparation.

It is interesting to note that the insertion of a fine cambric filter in a pump-oxygenator-kidney circuit does not result in an elevation of the blood flow to the level usually found in pump-lung-kidney preparations [Bayliss and Ogden, 1933], the presumption being that the filter does not remove the vaso-tonins. This observation suggests that the vaso-tonins which act upon renal blood vessels may be circulating in the blood of our heart-oxygenator preparations and that if there is any truth in our second hypothesis, the renal vaso-tonins and the substances responsible for early cardiac failure are not identical.

The term "detoxicated" defibrinated blood has been used for blood which has been passed through the lungs and thereby lost its vaso-constrictor properties, and we have seen there is abundant evidence that such a process occurs when the blood is tested on the isolated perfused kidney. That this mechanism of "detoxication" may prevent the onset

of early cardiac failure in the heart-oxygenator preparation is not denied but it has yet to be proved. Moreover, our observations raise the question as to the possibility of an additional mechanism being involved in the so-called "detoxication" of blood, namely one of mechanical filtration of particulate material in the blood.

SUMMARY.

1. An isolated mammalian heart-oxygenator preparation, in which the quantity and quality of work performed by each side of the heart can be varied at will, is described.

2. Early cardiac failure occurs in the heart-oxygenator preparation running on "fresh" defibrinated blood unless the blood is filtered through fine cambric filters before use and during circulation. Possible causes of this failure are discussed.

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THE INFLUENCE OF THE PARATHYROID ON THE METABOLISM OF CREATINE AND PHOSPHORIC ACID.

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There are reasons for associating the parathyroid glands with the metabolism of creatine phosphate in the muscles. It has been observed that the rate of resynthesis of creatine phosphate after stimulation of the muscles of thyroparathyroidectomized cats which have developed tetany is very much slower than in normal ones; also that the rate of this resynthesis in thyroparathyroidectomized cats which have recovered from tetany following the administration of parathormone, is similar to that observed in normal animals [Imrie and Jenkinson, 1932]. These observations, however, have been based on a comparison of results obtained from groups of animals. They would be more convincing if the effect of the parathormone could be demonstrated in the same animal.

Collip, Clark and Scott [1925] observed that the rise in serum calcium which follows the administration of parathormone reaches a maximum in 5–9 hours. It is conceivable therefore that the influence of this hormone on the creatine phosphate might be detectable within this limit of time. Accordingly some experiments have been carried out in order to test this hypothesis. In these, the rate of resynthesis of creatine phosphate after stimulation of the muscles was observed in one limb of a cat and several hours later similar observations were made on another limb. In some instances parathormone was given intravenously immediately after the muscles of the first limb were removed for analysis. If the hormone exerted any influence on the creatine phosphate within the time limits of the experiment, it should be apparent in the values obtained in the muscles of the second limb studied several hours later.

Experimental procedure.

Young cats were used. Thyroparathyroidectomy was performed under ether anæsthesia. The animals were permitted to recover and, after the symptoms of tetany were well-marked, the experiments were carried out.

Anæsthesia was induced with ether and maintained with chloralose. Immediately after the induction of anæsthesia the crural and sciatic nerves of one hindlimb were cut and the animal left for an hour to recover from the effect of the ether and from changes in the creatine phosphate which might arise from the muscular movements associated with the induction of anæsthesia. A portion of muscle was removed for examination and then the peripheral ends of the divided nerves were faradized for 10 min. The two electrodes employed were attached to the same induction coil. Analyses of the muscles were made at 15 or 20 min. intervals for an hour. Several hours later this procedure was repeated on the other hindlimb. The animals were kept under the influence of chloralose throughout the whole period. In some instances intravenous injections of parathormone were given immediately after the examination of the first limb was completed, in others no parathormone was given. The Eggletons' method [1929] for the phosphates was followed throughout except that the muscles were immersed in liquid air immediately after removal. It was found that the cat's muscle pulverized more readily when this procedure was adopted.

Results.

In Table I are set out figures for the creatine phosphate in the muscles of two thyroparathyroidectomized cats before and after stimulation. The corresponding graphs are shown in Fig. 1. The rates of resynthesis of

Table I. Creatine phosphate in the muscles of the hindlimbs of thyroparathyroidectomized cats before and after stimulation. The muscles of one limb were examined several hours after the other. Figures express creatine phosphate as mg. phosphorus per 100 g. muscle.

Exp. 1	Before stimulation	Zero	15 min.	30 min.	45 min.	60 min.
1st limb	42·5	19·9	22·2	25·0	28·9	33·9
2nd limb 6 hours later	40·6	17·6	20·0	22·2	25·8	28·9
Exp. 2			20 min.	40 min.	60 min.	
1st limb	43·5	17·5	21·6	23·6	27·5	
2nd limb 4 hours later	45·4	16·9	20·4	22·3	26·6	

creatine phosphate are strikingly similar in the separate limbs of each animal. The initial values for the creatine phosphate are lower and the rate of resynthesis after stimulation is much slower than that observed in normal cats [Imrie and Jenkinson, 1932].

If, however, parathormone is injected intravenously immediately after the muscles of the first limb have been removed for examination and observations on the second limb are made 4–7 hours later, the rate of resynthesis of the creatine phosphate after stimulation is more rapid than it was in the first limb where no parathormone had been given. The

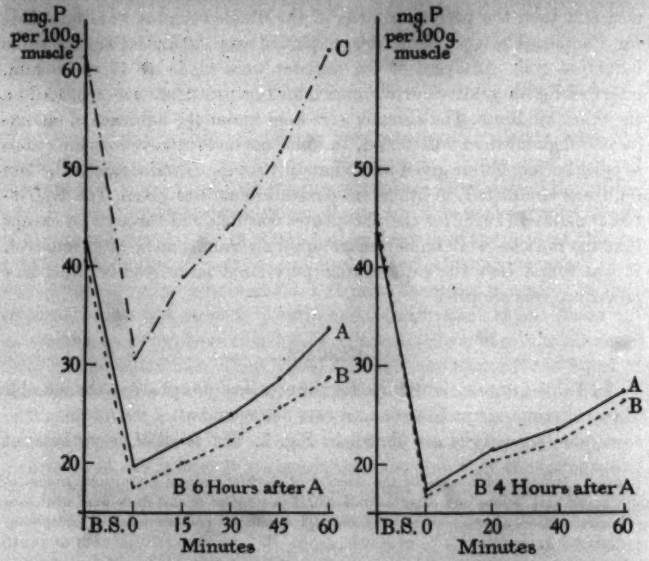

Fig. 1. Creatine phosphate in the muscles of thyroparathyroidectomized cats before and after stimulation. *A*, right hindlimb; *B*, left hindlimb; *C*, creatine phosphate in the muscles of a normal cat, similarly stimulated. Ordinates, mg. phosphorus in the form of creatine phosphate per 100 g. muscle; abscissæ, time: B.S., before stimulation; 0, immediately after stimulation.

results of four such experiments are included in Table II, and for one experiment are expressed graphically in Fig. 2. In each instance, though the initial values for the creatine phosphate have remained unaltered, the rate of the resynthesis of the creatine phosphate is more rapid after the parathormone has been given; in fact it resembles that observed in normal animals.

On two occasions, Exps. 7 and 8, creatine in amounts corresponding

to 200 mg. per kg. body weight was injected intravenously as well as the parathormone, after the muscles of the first limb had been removed for examination. The results are given in Table II.

TABLE II. Creatine phosphate in the muscles of the hindlimbs of thyroparathyroidectomized cats before and after stimulation. Immediately after the muscles of one limb were removed for examination 10 units of parathormone were injected intravenously and several hours later the muscles of the other limb were studied. In Exps. 7 and 8 creatine in amounts corresponding to 200 mg. per kg. body weight were injected along with the parathormone. Figures express creatine phosphate as mg. phosphorus per 100 g. muscle.

Exp. No.		Before stimulation	Zero	15 min.	30 min.	45 min.	60 min.
3	1st limb	53·8	29·4	32·7	37·0	37·6	38·8
	2nd limb	55·5	25·5	34·4	40·6	42·4	50·5
	6½ hours later						
4	1st limb	47·8	25·0	31·2	32·1	34·4	38·6
	2nd limb	49·0	24·5	36·1	39·0	43·4	47·5
	7 hours later						
				20 min.	40 min.	60 min.	
5	1st limb	47·1	19·4	24·4	25·2	35·4	
	2nd limb	51·5	17·6	37·0	37·6	49·4	
	5½ hours later						
6	1st limb	49·8	18·8	21·6	27·6	28·0	
	2nd limb	50·2	18·8	27·4	34·4	45·5	
	5 hours later						
7	1st limb	43·2	21·2	24·4	32·3	35·5	
	2nd limb	46·8	21·5	32·7	37·2	50·0	
	5 hours later						
8	1st limb	54·6	30·3	40·4	47·3	53·5	
	2nd limb	60·0	32·2	39·1	58·4	63·0	
	5½ hours later						

It is to be seen that the initial values for the creatine phosphate are slightly higher in the second limb than in the first. The curves of resynthesis of the creatine phosphate are similar to those obtained in cats which had received parathormone alone, except that in both instances the concentration of creatine phosphate in the muscles removed at the end of the hour was higher than it was before the muscles were stimulated. In Exp. 8 it is to be noted that the values for the creatine phosphate before and after stimulation of the muscles of the first limb are practically normal. This cat had its thyroids and parathyroids removed 2 days previously, but tetany was not well-marked and there was no dyspnœa at the time the experiment was begun. Nevertheless, the difference in the rate of resynthesis of the creatine phosphate after creatine and parathormone had been given is quite marked, and the final value for the creatine phosphate was higher than the initial one.

A few experiments of a different nature have been carried out in order to observe the creatine phosphate before and after stimulation in the muscles of normal cats which have been given creatine in the food with or without injections of parathormone for several days before the

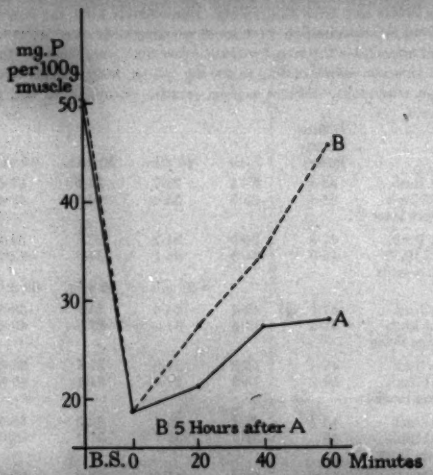

Fig. 2. Creatine phosphate in the muscles of thyroparathyroidectomized cats before and after stimulation. Parathormone injected intravenously after the muscles of the first hindlimb were removed for examination: the muscles of the second hindlimb were studied several hours later. *A*, right hindlimb before parathormone; *B*, left hindlimb after parathormone. Ordinates, mg. phosphorus as creatine phosphate per 100 g. muscle; abscissæ, time: B.S., before stimulation; 0, immediately after stimulation.

TABLE III. Creatine phosphate in the muscles of normal cats before and after stimulation. In Exps. 9 and 10, 5 g. of creatine were given daily for several days before the observations were made. In Exps. 11, 12 and 13, in addition to the creatine, parathormone in doses of 10 units was given subcutaneously as indicated. Figures express creatine phosphate as mg. phosphorus per 100 g. muscle.

Exp. No.	Days of treatment	Before stimulation	Zero	20 min.	40 min.	60 min.
9	9	62·0	17·7	38·3	46·7	62·4
10	7	59·5	17·5	28·9	43·0	58·1
11	9	68·2	22·0	34·8	40·8	55·4
12	8	69·0	17·3	33·4	49·4	52·0
13	3	*A 72·0	39·2	50·0	59·4	55·0
		*B 71·4	35·3	49·7	58·5	57·5

* In this experiment the muscles of both hindlimbs were examined, *B* 4½ hours after *A*.

observations were made. It is known that the creatine content of cat's muscle may be increased by feeding creatine [Folin and Denis, 1912], and that the creatine phosphate may also be increased when creatine is injected into cats which have received parathormone previously. In

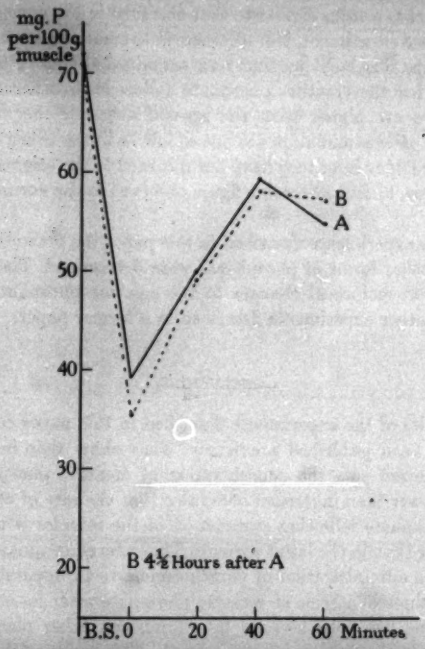

Fig. 3. Creatine phosphate in the muscles of a normal cat which had received 5 g. creatine + 10 units of parathormone for 3 days. *A*, right limb; *B*, left limb 4½ hours later. Ordinates, mg. phosphorus in the form of creatine phosphate per 100 g. muscle; abscissæ, time: B.S., before stimulation; 0, immediately after stimulation.

Table III are set out the results obtained in two experiments, Nos. 9 and 10, where creatine alone was given in daily doses of 5 g. for 7 and 9 days respectively. The initial values for the creatine phosphate and the rate of its resynthesis after stimulation of the muscle correspond to the average values shown in our former paper.

In three instances (Exps. 11, 12 and 13), where creatine in similar amounts was given along with daily doses of 10 units of parathormone, the initial values for the creatine phosphate are above the normal in every instance. After stimulation the creatine phosphate falls as in the normal animal and does not return to the original high level within the hour but reaches a value similar to that observed in the normal untreated animal. In one experiment, No. 13, where both creatine and parathormone were given, the muscles of one limb were examined 4½ hours after the other. The values for the creatine phosphate follow each other closely. The initial values are higher than the normal average: the values found immediately after stimulation are not so low as in the other experiments and at the end of an hour they have not returned to the original high level, but as in Exps. 11 and 12 reach a figure observed in the normal untreated animal.

In all the experiments described in this paper the orthophosphates as well as the other forms of phosphorus were determined. The orthophosphates showed reciprocal changes to the creatine phosphate. This was observed in other experiments described in a former paper.

Conclusions.

The results of the experiments described in this paper confirm those which have been published previously. They show that in thyroparathyroidectomized cats the concentration of creatine phosphate in the muscles is lower than in normal ones; also that the rate of resynthesis of creatine phosphate following stimulation of the muscles is much slower in the former than in the latter animals. They also show quite clearly that following the administration of parathormone to thyroparathyroidectomized cats the resynthesis of creatine phosphate after its concentration in the muscle has been lowered by stimulation takes place at a rate similar to that observed in normal animals. This has been demonstrated in the same animal in the course of individual experiments and is therefore more convincing than the results published previously which were obtained in groups of animals. It has also been shown that this effect of the parathormone is manifest within at least 5 hours of its administration intravenously.

Where creatine is administered along with parathormone in feeding experiments for some days or in acute experiments there appears to be a definite effect upon the initial values of the creatine phosphate in the muscles and the rate of its resynthesis after stimulation.

It is a pleasure to acknowledge our thanks to Prof. Leathes for his interest and advice.

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THE EXCRETION OF PROLAN AFTER INTRAVENOUS INJECTION INTO THE RABBIT.

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I. Introduction.

The abundance of ovary-stimulating substance ("Prolan") in the urine of pregnant women suggests that it is easily excreted, and also, unless it is present in the circulation in enormous quantities, that an appreciable proportion may be removed in this way. By contrast, the apparent absence of the substance from the urine of most lower mammals at all times of the œstrous cycle is remarkable, but little experimental work seems to have been carried out on the possibilities of excretion of the hormone by animals such as the rabbit. Smith and Moore [1933] were unable to demonstrate that any appreciable amount of the thyroid-stimulating hormone present in anterior lobe extract was excreted by guinea-pigs after subcutaneous injection. Snyder and Wislocki [1931] injected three rabbits with human urine of pregnancy, and subsequently tested the rabbit urine on other rabbits. Ovulation was obtained in one test animal. The present paper deals with more extensive experiments on these lines, carried out by a different technique and with a prolan extract of known potency.

II. Technique.

Prolan preparation. The same preparation of prolan was used throughout the experiments. It was stored as a dry powder at about − 2° C., other experiments having shown it to be indefinitely stable under these conditions. Appropriate amounts of the powder were made up into fresh aqueous solutions for each experiment. The potency of the extract had previously been determined by injection into groups of at least 20 normal œstrous rabbits. 10 mg. per rabbit caused ovulation in 98 p.c., while

5 mg. gave the effect in just over 50 p.c. 10 mg. has therefore been considered as the amount required to cause ovulation with comparative certainty.

Collection of urine. Since the time over which the urine had to be collected was comparatively short, it was possible to avoid the use of metabolism cages and thus to obtain the urine fresh and uncontaminated. Our technique was to open the abdomen under anæsthesia, empty the bladder, ligate the urethra, inject intravenously and, after the required time, to kill the animal, remove the bladder whole and drain into a measuring cylinder. Males and females were used for the excretion tests, but all animals were gonadectomized before injection.

Preparation of rabbit urine for injection. Since we aimed at using the whole of the urine collected from each injected rabbit some process of purification was necessary, but at the same time it was highly desirable to avoid loss of the hormone. Our technique was therefore to make up the urine to 80 p.c. alcohol by the addition of absolute alcohol, and to centrifuge off the precipitate and take it up in water. Even so, the resulting extract was found to be highly toxic unless other precautions were observed. All the urine extracts made from three rabbits injected soon after feeding killed the respective test animals. On the other hand, to secure adequate excretion during the experiment, it was necessary to give water after injection if food had previously been withheld[1]. One preparation made from a rabbit given only water on the day of the experiment was tolerated easily by the test animal. To avoid giving water to drink and to standardize the intake, our regular technique has been to give 100 c.c. saline intravenously at the time when the prolan was injected. By this means the total volume of urine excreted by the rabbit during the experiment, though very variable (20–80 c.c.), was kept about normal. We have no reason to suppose that the injection of saline altered the conditions of excretion of the hormone, and the one rabbit not given saline produced active urine.

Test animals. The rabbit urine extracts were tested by injection into œstrous rabbits; laparotomy was performed next day to ascertain whether ovulation had been induced.

III. Experimental results.

The amounts of prolan given to the rabbits were 30 mg. (3 exps.), 50 mg. (4 exps.) and 100 mg. (7 exps.), or about three, five and ten times the amount required to produce ovulation with certainty in an œstrous

[1] With a large ration of green stuff, water is not necessary in keeping rabbits.

animal. After prolan injection, urine was collected for times up to 9 hours, two samples (0–3 and 3–9 hours) being taken from one animal (EP 11). Even with the precautions mentioned above, collection for more than 9 hours produced highly toxic samples. The experiments are summarized in Table I, which also gives the results of the tests on the rabbit urine.

TABLE I. Excretion of prolan by the rabbit.

No. of exp.	Amount of prolan powder given (mg.)	Time urine collected after injection (hours)	Proportion of urine extract given to test animal	Result of test
EP 1	30	7	All	Negative
16	30	9	All	Positive
17	30	9	All	Negative
2	50	7	All	Negative
12	50	6	All	Negative
13	50	9	All	Positive
21	50	9	All	Positive
3	100	5	All	Positive
4	100	5	All	Negative
6	100	5¼	All	Positive
11	100	3	All	Positive
11	100	3–9	All	Positive
5	100	9	All	Positive
18	100	9	1/4	Positive
18	100	9	1/2	Positive
15	100	9	1/4	Negative

These results suggest the following considerations:

(*a*) Of two animals receiving 30 mg. (*i.e.* three ovulation doses), one gave active urine 9 hours later, so that rather less than one-third of the hormone appears to have been excreted during this time and recovered.

(*b*) Of the two tests on one-quarter of the urine obtained 9 hours after the injection of 100 mg. (*i.e.* ten ovulation doses), one was positive and the other negative. In other experiments, injection of all the urine and also of one-half of the urine collected 9 hours after injection of 100 mg. produced positive reactions.

These results again suggest that about one-third or less of the material is excreted in 9 hours. It would thus appear that the blood threshold, if any, which the hormone must attain before excretion can begin is comparatively low.

(*c*) The results suggest, though they do not prove, that excretion continues up to 9 hours. Thus, in both the 30 mg. and 50 mg. group, the urine appears to be more active after 9 hours than after 6 or 7 hours. Further, after the administration of 100 mg., active urine was obtained during the first 3 hours and also between 3 and 9 hours.

We have not determined what happens to the two-thirds of the hormone unaccounted for in the above results. Presumably some destruction takes place in the blood, and also excretion may well continue after 9 hours. Our results, however, do show that, given enough prolan in the blood, the kidneys of the rabbit can excrete sufficient in under 3 hours to cause ovulation in an œstrous rabbit, *i.e.* about 4 units per day per kg. body weight.

A pregnant woman excretes 500–1000 rabbit units of prolan per day, or roughly 10 units per day per kg. The amount which can be excreted by a rabbit is therefore of the same order as that which actually is excreted by a woman. It may thus be concluded that the failure of the pregnant rabbit to excrete prolan is due not to inability on the part of the kidneys, but more probably to lack of the hormone in the blood.

IV. Summary.

1. Prolan was injected intravenously into gonadectomized rabbits. About one-third of the hormone given was recovered from the urine within 9 hours. The amount excreted by these rabbits was of the same order (per kg. body weight) as the output of the pregnant woman.

2. The kidneys of the rabbit are, therefore, able to excrete appreciable quantities of the hormone, and their failure to do so during pregnancy is presumably due to lack of the substance in the blood.

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THE EXTRA-UTERINE SURVIVAL OF SPERMATOZOA.

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WHILE spermatozoa live in the epididymis of the male rat for about 42 days [Moore, 1928; White, 1932], their life is a matter of hours when they are introduced into the female genital tract. There are two main factors causing this shortened life—the violent activity which sperm display when dispersed in the accessory secretions of the male and the increased temperature to which they are subjected in the female. The question arises as to whether the uterus is the most favourable environment in the female for sperm survival or whether in addition to the two first mentioned factors the uterus itself exerts deleterious effects. Although Kugota [1929] reported that the uterine secretions of the rat during œstrus had a beneficial effect on sperm survival *in vitro*, to test this point adequately it is necessary to confine sperm in another cavity where they may be subjected to conditions approximating to those in the uterus. The abdominal cavity is unfavourable [Hoéhne and Behne, 1913]. Accordingly sperm were injected into the lateral ventricle of the brain. Here they are freely motile, are exposed to the same temperatures as in the uterus, and may readily be recovered for examination. Data on the survival of sperm in this environment will be given in the present paper.

MATERIAL AND METHODS.

Thirty-four female rats in œstrus or pro-œstrus and twenty-one males were used in this work. The technique for injecting the sperm into the lateral ventricle was briefly as follows: Under ether anæsthesia a median incision was made in the skin over the calvarium. The fascia was removed from a small area over the parietal bone and a hole 1 mm. in diameter trephined approximately 3 mm. posterior to the fronto-parietal suture and 2 mm. lateral to the sagittal suture. Sperm were removed from the tail of the epididymis or ductus deferens and mixed with a small quantity of

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Baker's solution or Tyrode's solution. With the aid of a very fine flatly ground hypodermic needle 0·02–0·03 c.c. of this sperm suspension were injected into the lateral ventricle through the hole in the skull. No bleeding usually resulted. This seems to be an important point, since in the few cases where slight hæmorrhage occurred negative results were obtained. It is necessary to direct the needle at the proper angle and only to the correct depth in order to penetrate the ventricle, which is little more than a potential cavity.

If more than 0·05 c.c. of solution is injected into the ventricle the animals sometimes show symptoms of cerebral pressure after coming out of the anæsthetic. For this reason only about half this quantity, which is sufficient for recovering large numbers of spermatozoa, was injected. After the appropriate interval the animals were killed by decapitation and the condition of the sperm determined by laying the ventricle open and removing the contents with a small pipette for microscopical examination.

For the injection of sperm into the uterus Yochem's [1929] technique, which consisted of ligating the uterus above the cervix and injecting the sperm suspension into both horns, was followed. Although he injected $\frac{1}{4}$ c.c. into each horn, 0·1 c.c. seems to contain ample sperm and does not put the œstrous uterus, already distended with fluid, under undue pressure.

Moore's [1928] method for quantitatively estimating the degree of motility was used. Maximum motility by this system is ****, meaning that all sperm are violently motile. When all but a few sperm are considerably motile the designation is ***. Likewise when only half are somewhat motile the reading is **, and when only a few are still motile, even one or two, the designation is *. Total absence of motility is 0. Normal sperm from the tail of the epididymis or ductus deferens always show **** motility when mixed with Tyrode's or other physiological solutions.

Results.

Motility has been detected in sperm from the lateral ventricle of the brain on one occasion 17½ hours after injection and frequently after 15 hours (Table I). Sperm from the tail of the epididymis seemed to survive longer than those from the ductus deferens, which is also the case in the male after epididymal isolation [see White, 1932]. Since the animals had not copulated for some time before the experiments were begun, they undoubtedly had older and less vigorous sperm in the distal

TABLE I. The survival of spermatozoa in the lateral ventricle of the rat brain.

Animal	Time after injection hours	Sperm taken from	Motility	Number of sperm recovered
B 1	4½	Ductus deferens	0	Very few
BH 2	4½	Tail epididymis	*	Many
BH 3	7½	Ductus deferens	*	,,
B 44	10	,, ,,	0	Very few
B 61	11	,, ,,	0	,,
W 91	11½	Tail epididymis	*	Many
B 64	12½	,, ,,	*	,,
W 28	12½	,, ,,	*	,,
B 53	15	Ductus deferens	0	Few
W 30	15	Tail epididymis	*	Many
G 00	15	,, ,,	*	,,
W 33	15	,, ,,	*	,,
W 12	17½	,, ,,	0	,,
BH 99	17½	,, ,,	0	,,
B 00	17½	,, ,,	0	,,
BH 4	17½	,, ,,	*	,,
W 21	17½	,, ,,	0	,,
B 45	18	,, ,,	0	,,
GH 43	18	,, ,,	0	Very few
W 4	18	,, ,,	0	Many

end of the ductus deferens than in the epididymis. In a single case Yochem [1929] found motile sperm 12½ hours after injection into the uterus of an œstrous rat. None were found after longer periods. The writer has been unable to recover motile sperm for longer than 10 hours after injection into the uterus, although a number of animals were examined (Table II).

TABLE II. Survival of spermatozoa injected into uterus of œstrous rat*.

Animal	Time after injection hours	Motility	Number of sperm recovered
7	5	*	Few
8	5	0	,,
10	7½	*	Very few
9	10	0	Few
11	10	*	Many
13	10	0	,,
14	10	0	Few
5	12½	0	None
6	12½	0	Few
1	15	0	Very few
2	15	0	Few
3	17½	0	,,
4	17½	0	Many

* In this series all sperm for injection were removed from the tail of the epididymis.

The conditions after the injection of sperm are possibly not altogether comparable to those after normal copulation, since the sperm were not mixed with the accessory secretions of the male. It was desired to obtain data on sperm survival after copulation, but several attempts to get

timed matings at night by observing the animals or examining the females at 15 or 30 min. intervals were unsuccessful. Yochem was more fortunate. He found motile sperm after 12 hours in the uterus, after 16 and 17 hours in the oviducts, and after 14½ hours in the vagina. This relatively long survival in the vagina was probably due to the fact that rats usually form copulation plugs. Sperm confined in such a plug are doubtless protected from the vaginal secretions which in most mammals are very unfavourable for sperm survival.

Discussion.

Yochem [1929] found motile sperm in the Fallopian tube 17 hours after mating, but in the uterus for only 12 hours. Likewise motile sperm have been recovered from 15–17½ hours after injection into the lateral ventricle of the brain. The longer survival in the tubes than in the uterus may be due either to a more favourable environment there, or to the more vigorous sperm leaving the uterus and reaching the Fallopian tube.

The shorter survival of sperm in the uterus than in the lateral ventricle of the brain, where they are often motile for 15 hours after injection, indicates that the brain cavities are possibly more favourable for the survival of highly motile sperm. This suggests that, at least in the case of the rat, the uterus is not specifically adapted for the longest possible survival of motile sperm. The fact that the uterus is slightly less suitable may be due to the presence of leucocytes, many of which are found even in the œstrous uterus, or to the presence of unfavourable secretions.

Summary.

A comparison of the survival of sperm in the uterus and in another body cavity, the lateral ventricle of the brain, has been made.

Sperm have survived in the brain for 17½ hours, 5 hours longer than in the uterus.

It is concluded that if the uterus is not antagonistic, it is at least not specifically favourable for the maximum survival of highly motile sperm.

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HISTAMINE IN CANINE GASTRIC TISSUES.

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In 1905 and 1906 Edkins reported that aqueous extracts of pyloric mucosa, when injected subcutaneously, caused secretion of gastric juice in the dog. Extracts of fundic mucosa did not stimulate gastric secretion, although they were stated to be more potent in lowering blood-pressure than the pyloric extracts. As a result of this work, the second or chemical phase of gastric secretion is now believed by many to be due to the formation in the pyloric mucous membrane of a specific substance called "gastrin," which when absorbed into the blood stimulates the gastric glands.

In 1920 Popielski demonstrated that histamine caused gastric secretion, and since then there has been considerable speculation as to whether histamine and "gastrin" are identical. In 1932 Sacks, Ivy, Burgess and Vandolah reported the isolation of histamine from hog pyloric mucosa. They showed that, after incubation with histaminase, saline extracts of this tissue no longer produced gastric secretion. They concluded that if a "gastric hormone" has been extracted from the pyloric mucosa the active principle is histamine. These workers consider the possibility that the "gastric hormone" may not as yet have been extracted from the mucosa. In these studies no reference was made to the investigation of any part of the stomach other than the pyloric mucosa.

In view of this similarity between "gastrin" and histamine it seemed advisable to study the distribution of the amine in various parts of normal stomachs. For this purpose dogs, after 16 hours' starvation or in some cases after a meat meal, were used. The animals were killed instantly by means of a captive-bolt pistol, the stomach was removed immediately, wiped as clean as possible with dry gauze, and then dissected into pyloric mucosa and muscle, and fundic mucosa and muscle. Each section was then finely minced, and weighed portions used for histamine determina-

tions. Extracts were prepared by our customary hydrochloric acid procedure [Best and McHenry, 1930]. The minced tissue was thoroughly mixed with hydrochloric acid within 30 min. after the death of the dog, and it is unlikely that any change occurred in the histamine content of the tissue in the interval, especially since the gastric tissues of the dog do not contain histaminase in amounts detectable by the methods available [Best and McHenry, 1930]. The depressor material present was determined by assay against a standard histamine solution, using the blood-pressure method in an etherized cat. Table I shows the depressor contents calculated as milligrams of histamine per kilogram of tissue.

TABLE I. Histamine content of dogs' stomachs (mg./kg.)

		Pyloric		Fundic	
No.	Condition	Mucosa	Muscle	Mucosa	Muscle
831	24 hr. starvation	28	17	48	16
856	24 ,,	40	4	60	14
857	30 ,,	50	15	90	33
869	24 ,,	37	19	80	27
	Average	39	14	69	22
842	1 hr. after meat meal	54	18	72	25
858	2 ,,	100	50	110	60
860	1½ ,,	46	30	80	28
861	2 ,,	54	15	105	38
	Average	63	28	92	38

A number of depressor substances, in addition to histamine, may be present in these tissues. Extracts prepared by acid digestion are free from adenylic acid and related compounds, and from "callicrein," since such treatment destroys these substances. All extracts were assayed before and after atropinization of the cat. Since these two assays were always identical it may be stated that the solutions did not contain choline or acetylcholine in amounts sufficient to be detectable by this procedure. However, an etherized cat does not respond to injections of small amounts of choline, and more sensitive methods have shown this compound to be present. Assay of the solutions against acetylcholine on isolated rabbit intestine showed small amounts of choline compounds to be present, and this result was confirmed by assays after acetylation of the solutions. These assays were kindly performed for us by Mr O. M. Solandt.

Incubation of the solutions with added histaminase under standard conditions as to *p*H and temperature [McHenry and Gavin, 1932] caused a disappearance of all the depressor activity. It has not yet been demonstrated that this enzyme is specific for histamine, but it does not

affect several related amines and iminazole compounds or choline. Incubation without histaminase did not alter the depressor effects of the gastric extracts.

Previous work in this laboratory showed that histamine was not formed by the acid digestion procedure. Pure egg albumin and pure casein, 2 g. in each case, were carried through the process and the resultant extracts showed no depressor action. Solutions of *l*-histidine dichloride were similarly treated with the same result. After adding *l*-histidine dichloride to minced stomach, the mixture was extracted, and no increase was found in the depressor content of the final extract when compared with controls from the same lot of tissue. However, this procedure might have liberated the amine from a loosely bound complex in which it already existed in the decarboxylated form. Simple saline extracts of gastric tissues were found to contain a depressor substance which was similar to that present in the extracts resulting from acid digestion, and these saline extracts likewise lost their depressor effect when incubated with histaminase.

Although these extracts of gastric tissues contained small amounts of choline compounds, the evidence cited in the above three paragraphs justifies the conclusion that all the depressor activity measured by the etherized cat procedure was due to histamine.

Table II gives the average total amounts of histamine present in each type of tissue. Not only does fundic mucosa contain more histamine per

Table II. Histamine content of a fasting dog's stomach.

Tissue	Weight g.	Amount of histamine mg.	P.c. of total
Pyloric mucosa	15	0·6	12
Pyloric muscle	8	0·1	2
Fundic mucosa	60	4·1	80
Fundic muscle	15	0·3	6
	Total	5·1	

kilogram than does pyloric mucosa, but there is much more of the former tissue in the stomach. A surprisingly large amount, 80 p.c. of the total histamine content of the stomach, was present in the fundic mucosa, while only 12 p.c. was found in the pyloric mucosa. If the suggestion of Sacks, Ivy, Burgess and Vandolah is correct, that "gastrin" is histamine, then the fundic mucosa is probably a much more important source than the pyloric section. Muscle from either part of the stomach contains little histamine.

Since Edkins has stated so definitely that fundic extracts do not produce secretion, and this has received general acceptance, it seemed essential to carry out experiments on this point. For this work a dog with a gastric fistula was prepared for us by Dr Best. This dog has been given a number of solutions subcutaneously, and the stomach contents have been collected at 5-min. intervals after the injection, by means of a catheter inserted through the fistula. The results of these experiments are summarized in Table III. Simple saline extracts of fundic mucosa,

TABLE III. Secretagogue effects of gastric extracts.

Injected solution		Volume of gastric juice collected in 30 min.
Volume and nature	Histamine content mg.	c.c.
0·22 c.c. histamine standard	0·2	40
0·5 c.c. histamine standard	0·5	40
50 c.c. solution prepared by HCl digestion of fundic mucosa	0·8	50
200 c.c. saline extract of fundic mucosa	0·7	50
90 c.c. saline extract of pyloric mucosa	0·2	20
70 c.c. saline extract of fundic mucosa	0·4	60
70 c.c. solution from same lot incubated with histaminase	0 (formerly 0·4)	None
70 c.c. solution from same lot incubated without histaminase	0·4	60
30 c.c. of same lot after 6 days in refrigerator	0·2	20
40 c.c. saline extract of pyloric mucosa incubated with histaminase	0 (formerly 0·2)	None
60 c.c. saline	0	None

and solutions prepared from similar tissue by acid digestion, definitely caused gastric secretion. Their power to do so was destroyed by incubation with histaminase, while a control experiment showed that the enzyme, and not simply incubation, was the inactivating factor. Histaminase similarly inactivated an extract of the pyloric mucosa, thus confirming Ivy's results. In all cases disappearance of secretagogue powers was coincident with a loss of depressor activity. As indicated above, this latter effect was due to the presence of histamine. Since the possibility existed that the dog might have developed a conditioned reflex, an injection of saline was given, but this failed to evoke secretion.

SUMMARY.

1. Histamine is contained in relatively large amounts in the mucous membrane of the stomach of the normal dog and in smaller quantities in the gastric muscle. Of the total amount of this substance contained in a stomach, approximately 80 p.c. is present in the fundic mucosa.

2. Extracts of dog's fundic mucosa, as well as those from pyloric tissue, have secretagogue powers which are lost after incubation of the solutions with histaminase. The results of Sacks, Ivy, Burgess and Vandolah on pyloric mucosa are thus confirmed and, in addition, it is shown that the fundic mucosa contains more secretagogue material than the pyloric. This result does not support the findings of Edkins.

This investigation was carried out in the laboratory of, and under the direction of, Prof. C. H. Best, to whom and to Dr J. G. FitzGerald, Director of the School of Hygiene, we are greatly indebted.

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THE EFFECT OF ATROPINE ON ADRENALINE HYPERGLYCÆMIA IN RABBITS DECEREBRATED ANTERIOR TO THE PONS.

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The hyperglycæmia which results from decerebration, like that following Claude Bernard's piqûre, is supposed by some to be nothing more than a form of adrenaline hyperglycæmia due to stimulation of the adrenal glands through the splanchnic nerves. This possibility has been discussed elsewhere in the light of experiments in which it was tested by seeing whether hyperglycæmia still follows decerebration or piqûre after removal of the adrenal glands or section of the splanchnic nerves (cf. Macleod and Pearce [1912], Donhoffer and Macleod [1932], Evans, Tsai and Young [1931]). As a rule, under these conditions the blood-sugar level fails to rise, but such a result does not necessarily prove that increased secretion of adrenaline is alone responsible for the hyperglycæmia which occurs with the adrenals intact. Stimulation of glycogenolysis in the liver through the nerves supplying this organ may also be interfered with by these operations, and the possibility must not be lost sight of that a sudden cessation in the secretion of insulin may follow decerebration or piqûre. As a matter of fact, decerebration may be followed by hyperglycæmia when it is performed on an adrenalectomized animal which has been kept alive by injections of cortical extract, provided glycogen has been allowed to accumulate in the liver, as a result of rich feeding with carbohydrates.

It seemed that further evidence bearing on these questions might be obtained by a closer comparison of the types of hyperglycæmia caused by adrenaline and by decerebration, and particularly, by seeing whether they both respond in the same way to the injection of atropine. Donhoffer and Macleod showed that this drug prevented the rise in the blood-sugar percentage which otherwise occurs after pontine decerebration in rabbits in which, by previous fasting, the glycogen content of

the liver has been reduced to a low level (below 1·0 p.c.). Others have also shown that atropine suppresses or, especially when given along with ergotamine, may prevent the rise in the blood-sugar level following oral administration of sugar, but that it has no effect on the hyperglycæmia which follows the direct intravenous injection of sugar.

In view of these results it seemed worth while to compare the effects of atropine on decerebration hyperglycæmia and on that produced by injection of adrenaline and by intravenous injection of sugar. As a preliminary it was, of course, necessary to determine both the dose of adrenaline and the amount of glucose which would cause, by continuous intravenous infusion, a degree of hyperglycæmia comparable with that observed after pontine decerebration in fasted rabbits.

Methods.

Rabbits which had fasted from 16 to 24 hours after being anæsthetized by intravenous injection of amytal were decerebrated in Region 2 by Schmidt's method [1923], one of the carotid arteries being left untied. At the beginning and end of the experiment a hindleg was amputated and plunged into a saline freezing mixture at a temperature below −10° C. After an interval greater than 15 min. a representative muscle sample was cut from the frozen limb and placed directly into hot KOH for the estimation of glycogen by the Pflüger method. Liver samples for glycogen estimation were taken at the beginning and end of the experiment and placed directly in hot KOH. Blood samples were withdrawn, generally from the carotid artery but sometimes from an ear vessel, at half-hourly intervals, for estimation of glucose by the Hagedorn-Jensen method. To control any possible effect of the injection of fluid on the blood-sugar level, normal saline was injected into the jugular vein at a constant rate for some time following decerebration. When the temporary post-operative hyperglycæmia had subsided and the blood-sugar concentration had reached a relatively steady value, the saline perfusion was replaced by one of adrenaline or of glucose, the actual rate of the administration of fluid being unaltered.

Adrenaline was injected in amounts varying from 0·000104 to 0·00079 mg. per kg. per min.

Glucose was injected in aqueous solution in amounts of from 0·395 to 0·571 g. per kg. per hour in concentrations of from 8–20 p.c. In one group of experiments atropine, in aqueous solution, was administered intravenously in single doses.

Results.

The injection of saline solution alone did not have any effect on the blood-sugar level. This can be seen from the curves shown in Fig. 1, in which the results of four of the adrenaline experiments are plotted. In all of them saline solution alone was injected at a constant rate during the two hours immediately following the decerebration, which was made

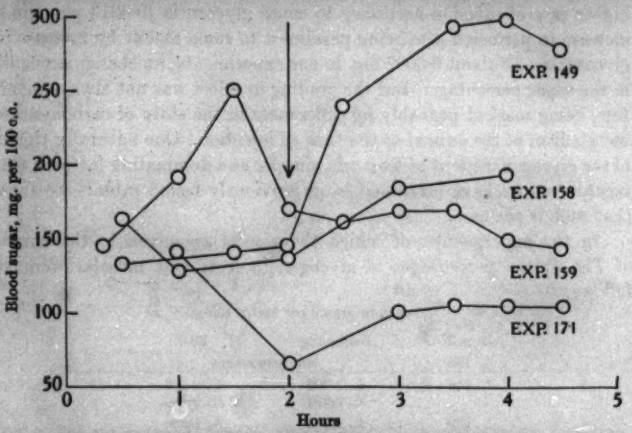

Fig. 1. The effect on the blood-sugar level of injecting adrenaline by continuous intravenous perfusion at varying rates into rabbits decerebrated in Region 2. At arrow, saline injection was replaced by adrenaline.

Rate of injection of adrenaline:

Exp. 149.	0·00025	mg. per	kg. per min.
Exp. 158.	0·000214	,,	,,
Exp. 159.	0·00018	,,	,,
Exp. 171.	0·000192	,,	,,

anterior to the pons. In three experiments the blood-sugar level remained undisturbed by this operation and did not rise during the saline transfusion; in one experiment the decerebration caused an immediate rise in the level—probably because of accidental injury to the pons—but this was only temporary, the normal being regained by the third hour.

That saline injections have no hyperglycæmic effect was also noted in all the experiments in which glucose was subsequently injected. It will be convenient to consider the adrenaline experiments first.

The effect of the addition of adrenaline to the saline is illustrated in the curves of Fig. 1, which show that a degree of hyperglycæmia comparable with that which follows pontine decerebration is produced by 0·00025 mg. adrenaline hydrochloride per kg. body weight and per minute. According to the curves, it would also appear that progressively smaller doses than this have correspondingly lesser effects on the blood-sugar level. Trendelenburg and Fleischhauer [1913] found a somewhat higher rate of infusion necessary to cause glycosuria (0·0013 per min.), increase in perfusion rate being paralleled to some extent by increase in glycosuria. At about 0·0002 mg. in our experiments, no change occurred in the sugar percentage. But the grading in effect was not always manifest, being masked probably by differences in the state of carbohydrate metabolism of the animal at the time of injection. One naturally thinks of the glycogen content of liver and muscles as a dominating factor in this regard, but we have been unable, in previously fasted rabbits, to show that such is the case.

In the experiments, of which the results are given in the curves of Fig. 1, the percentages of glycogen in liver and muscles were as follows:

Glycogen in grams per 100 g. tissue.

Exp. No.	Beginning	End
148	No estimations	
158	L. 0·902	L. 0·504
	M. 0·237	M. 0·230
159	L. 1·214	L. trace
	M. 0·861	M. 0·570
171	L. 1·318	L. trace
	M. 0·269	M. 0·259

The percentages at the start happened to be much alike for the liver, but very variable for the muscles. No conclusions can be drawn from these results regarding the behaviour of the glycogen during the periods of adrenaline injection.

Although 0·00025 mg. adrenaline usually produces marked hyperglycæmia, it may fail to do so in certain animals and we are unable to account for the difference. Thus in two experiments the following results were obtained:

	Exp. 1	Exp. 2
Average blood sugar during saline injection	0·160 p.c.	0·120 p.c.
Average blood sugar during adrenaline injection	0·090 p.c.	0·140 p.c.

The dose of adrenaline in Exp. 1 was 0·00029 mg., increased later to 0·00040 mg. In Exp. 2 the rate of injection was 0·0004 mg. per kg. per

min. It is considered permissible, however, to conclude that 0·00025 mg. adrenaline (hydrochloride) per min. and per kg. body weight will usually produce, in previously fasted rabbits, a degree of hyperglycæmia, which is comparable with that following pontine decerebration, but it must be admitted that the occurrence of exceptions detracts somewhat from the

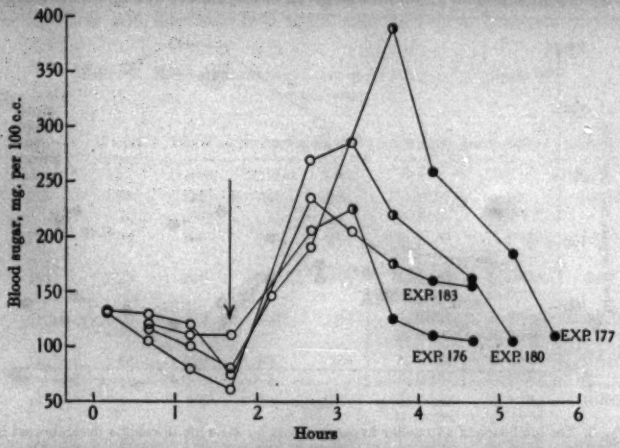

Fig. 2. The suppression of adrenaline hyperglycæmia by atropine in rabbits decerebrated in Region 2. The arrow indicates the beginning of adrenaline infusion in place of saline. Blood-sugar values after intravenous injection of a single dose of atropine are shown in black.

Rate of adrenaline injection:

Exp. 176.	**0·000486**	**mg. per kg. per min.**
Exp. 177.	**0·000332**	**„**	**„**
Exp. 180.	**0·000405**	**„**	**„**
Exp. 183.	**0·00072**	**„**	**„**

value of some of our later results on the effect of atropine (*e.g.* those shown in Fig. 3).

In order to study the effect of atropine, two methods were employed: (1) injecting it after adrenaline hyperglycæmia had become established and (2) injecting it at the same time as adrenaline.

The results of four experiments of the former type are shown in Fig. 2. In two of them atropine caused the blood-sugar percentage to fall rapidly. The glycogen percentages in the liver and muscles did not

differ in their behaviour from those in the experiments in which adrenaline alone was injected, the percentage of liver glycogen being over 1·5 to start with in all cases.

The results of two experiments in which atropine was injected in large dosage at the commencement of the adrenaline injections are shown along with one in which no atropine was given in the curves of Fig. 3. Although

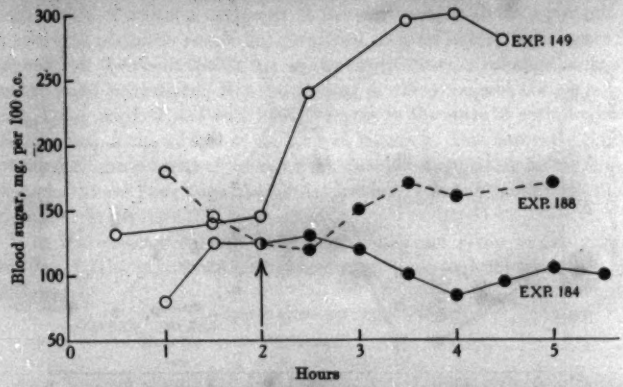

Fig. 3. The inhibition of adrenaline hyperglycæmia by atropine in rabbits decerebrated in Region 2. The arrow indicates the substitution in all three experiments of the beginning of adrenaline infusion in place of saline. It also indicates the intravenous injection of a single dose of atropine (5 mg. per kg.) in two experiments. The post-atropine blood-sugar values are shown in black.

Rate of injection of adrenaline:

Exp. 149. 0·00025 mg. per kg. per min.
Exp. 188. 0·00044 „ „
Exp. 184. 0·00079 „ „

a moderate increase in the blood-sugar percentage occurred in one of the experiments, the general effect of atropine was to inhibit the hyperglycæmic action of adrenaline, but these results are not so convincing as those shown in Fig. 2, for the reason already given. Glycogen was determined in the liver and muscles of Exp. 188, with the following results:

Liver: start 1·34 p.c.; finish, trace.
Muscles: start 0·24 p.c.; finish 0·30 p.c.

The effect of the continuous injection of glucose solutions is shown in abbreviated form in Table I. By comparing the averages of the blood-

sugar percentages before and during injection of glucose, it can be seen that 0·4 g. per kg. body weight and per hour had no effect, whereas a decided rise occurred when 0·45 g. or more was injected. Between these two amounts it is possible that a grading in effect could be demonstrated, but our results within this range are not sufficiently numerous to make the comparison. The table (Table I) also gives the percentages of glycogen in the liver and muscles. It is significant that there is no relationship

TABLE I. Effect of continuous intravenous injections of glucose at varying rates.

Mean blood sugar during saline	Wt. of animal kg.	Blood sugar during glucose			Glycogen percentages		Rate of injection g. per kg. per hr.
		Lowest	Highest	Mean	Before	After	
155	2·42	135	250	184	L. 0·040 M. 0·441	L. Nil M. 0·224	0·413
172	2·08	280	310	294	L. 0·548 M. —	L. 0·080 M. —	0·571
180	1·35	240	310	275	L. 0·587 M. 0·500	L. 0·346 M. 0·273	0·501
213	1·70	320	400	366	L. 0·807 M. 0·397	L. 2·779 M. 0·270	0·460
142	1·95	210	300	255	L. 0·045 M. 0·497	L. 2·328 M. 0·297	0·517
160	1·5	115	220	169	L. 2·091 M. 0·398	L. 1·747 M. 0·318	0·395
185	1·27	115	220	165	L. 1·726 M. 0·357	L. 0·138 M. 0·053	0·399
246	1·37	270	330	305	L. 4·518 M. 0·439	L. 1·123 M. 0·295	0·448

between the behaviour of the liver glycogen and the amount of glucose injected. Thus, the percentage of glycogen diminished in four of the experiments in which sufficient glucose was injected to raise the average percentage of blood sugar while it increased in two of them. It diminished in the two experiments in which there was no rise in the blood-sugar level. The muscle glycogen became reduced in percentage in all of the experiments and no relationship is apparent between the extent of this reduction and the rate of sugar injection.

To investigate the effect of atropine on this form of hyperglycæmia, large doses of the alkaloid were injected after the sugar had been transfused for some time. It can be seen from the curves of Fig. 4 that this had no effect on the blood-sugar level. Glycogen estimations were made in liver and muscle as usual. In all three cases, the liver glycogen rose

from 1 to 3 p.c. The muscle glycogen did not bear any relationship to that of the liver, sometimes rising and sometimes falling.

When atropine was injected during the whole of the glucose perfusion, the results were inconstant and no conclusions are drawn from them.

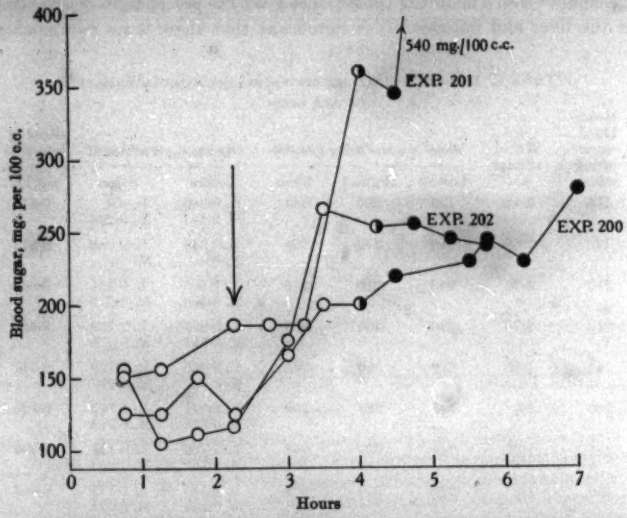

Fig. 4. The effect of atropine on the hyperglycæmia produced by the continuous intravenous perfusion of glucose into rabbits decerebrated in Region 2. The arrow indicates the beginning of infusion of glucose solution in place of saline. The post-atropine blood-sugar values are shown in black.

Rate of injection of glucose:

Exp. 200. 0·363 gm. per kg. per hour.
Exp. 201. 0·622 „ „
Exp. 202. 0·664 „ „
Atropine injection: 5 mg. per kg. in single dose.

Summary.

It is undoubtedly of some significance that atropine should affect the hyperglycæmia which results from injections of adrenaline much in the same way as it does that which follows pontine decerebration or the oral

administration of sugar. If these effects were due to a retardation in the rate of glycogenolysis in the liver or to an increase in glycogen formation in this organ, it might be expected that this would be detected by a less marked decrease in the glycogen content of the organ during the course of the atropine experiments than occurred in those in which no atropine was given. But the behaviour of the glycogen content is too irregular even in the experiments without atropine to justify any conclusions being drawn from its behaviour when this was given. It would also be reasonable to suppose that if atropine promoted the formation of liver glycogen in these experiments, it would also do so when given at the same time as glucose was being added to the blood by intravenous injection, but, here again, the glycogen results are too inconstant to be of value. Even when glucose alone was added to the blood, the liver glycogens, in a long series of experiments, did not behave alike. Such results confirm the impression which is gaining ground that the conditions which determine the behaviour of glycogen in the liver are much more complex than many have supposed them to be.

It is interesting to note that the amount of glucose necessary to cause hyperglycæmia by intravenous injection in fasted rabbits decerebrated anterior to the pons is between 0·4 and 0·45 g. per kg. per hour. This is considerably less than that given by Cori [1931] for rabbits under amytal anæsthesia, viz. 2 g. The dose of adrenaline necessary to cause in rabbits decerebrated anterior to the pons a degree of hyperglycæmia comparable with that which follows pontine decerebration was usually found to be 0·00025 mg. per kg. per hour, but in a few of the experiments of this group, larger doses were required. Cori found that 0·00005 mg. per kg. per hour caused a rise in the blood-sugar level in unanæsthetized rabbits.

Conclusions.

1. In rabbits deprived of food overnight and then decerebrated anterior to the pons, the amount of adrenaline hydrochloride necessary to cause by continuous intravenous injection a degree of hyperglycæmia comparable with that which follows pontine decerebration, is usually 0·00025 mg. per kg. per hour. Sometimes, however, larger doses fail to have this effect.

2. Atropine injected after adrenaline hyperglycæmia has become established may cause the blood-sugar level to fall rapidly. When the alkaloid is given in one large dose at the start of the adrenaline injections, it appears to retard the rise in the blood-sugar level.

3. When glucose is added to the blood by continuous intravenous injection, it requires 0·4–0·45 g. per kg. per hour to cause a definite rise in the blood-sugar level, and atropine has no influence on this form of hyperglycæmia.

The behaviour of the glycogen of the liver and muscles in the present experiments was too inconstant to permit of any conclusions being drawn.

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THE ALLEGED OCCURRENCE OF ACETYLCHOLINE IN OX BLOOD.

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In spite of the closest and friendliest cooperation between Prof. Kapfhammer and ourselves, we are still unable to reach agreement on the question of the alleged occurrence of physiologically important amounts of acetylcholine in normal blood.

In a long series of experiments we have consistently failed to detect physiologically more than the merest traces of activity resembling that of acetylcholine (equivalent to not more than 0·1 mg. of acetylcholine chloride per litre of blood) in blood extracts at any stage in the process of purification, while he and his collaborators describe the detection and chemical identification of amounts up to as much as 40 mg. per litre.

It appears to be desirable to report the results of our final attempt to clear up the discrepancy, since, having regard to the present state of the literature on this question, our silence might reasonably suggest that we no longer maintained our original position.

About a year ago, a full and friendly exchange of information and experiences having failed to disclose the reason for our contradictory findings, at the invitation of Prof. Kapfhammer I went to Freiburg in the hope of discovering, by collaboration with him in his own laboratory, the explanation of our discordant results.

I will state forthwith that I had no difficulty in isolating acetylcholine from the two samples of blood examined during my visit to Freiburg. Ox blood was collected on two occasions from the municipal slaughter-house. Dr C. Bischoff and I each worked up 1 litre of each specimen according to the Freiburg technique. This consists in precipitating the blood proteins with alcohol, and treating the extract, after concentration, with trichloroacetic acid to remove residual protein. Fat and excess of trichloroacetic acid are then removed by extraction with ether.

After appropriate concentration of the extract acetylcholine is precipitated with "Reinecke" acid, the "reineckate" is decomposed and the base converted into chloroaurate for identification.

At each stage of the process Dr Bischoff's and my results were identical in both experiments.

The two experiments were particularly remarkable in that the physiological behaviour of the acetylcholine associated with the first differed strangely from that of the acetylcholine associated with the second.

The first sample of blood was taken to the stage of purification with trichloroacetic acid before it was tested for physiological activity. The result of this test was quite concordant with our own experiments at Hampstead, for it displayed only a minute activity, equivalent to 0·1–0·5 mg. of acetylcholine chloride per litre of blood. Nevertheless, we proceeded to the next stage, namely precipitation with Reinecke acid, and most surprisingly the solution of chlorides derived from the precipitated "reineckate" possessed physiological activity equivalent to 40 mg. of acetylcholine chloride per litre of blood. There was no doubt that this activity was in fact due to acetylcholine, since the chloroaurate of the base was isolated and identified chemically.

The second sample of blood was precipitated with alcohol, and after 1 hour the alcoholic filtrate was tested physiologically, at Dr Bischoff's suggestion, before concentration on both the cat's blood-pressure and the rabbit's isolated intestine. Although the alcohol interfered to some extent in the second method, an unmistakable acetylcholine effect was observed in both, indicating approximately 22 mg. of acetylcholine chloride per litre of blood. Assayed subsequently at the trichloroacetic acid and "reineckate" stages, this extract gave equivalents of 27 and 25 mg. respectively, and acetylcholine was finally isolated and identified as the chloroaurate.

Thus acetylcholine was actually isolated from the two blood extracts and identified chemically. The appearance of the active base at the end of the fractionation of the first extract, which up to that point had given no indication that it contained more than a trace, was uncanny; particularly as the second extract displayed at every stage of purification the typical physiological activity in a degree corresponding to the amount of acetylcholine eventually isolated.

It is true that Bischoff, Grab and Kapfhammer [1931] have reported that alcoholic extracts of blood are sometimes obtained which give no physiological indication of the acetylcholine which they actually

contain until they have been treated with trichloroacetic acid, the presumption being that acetylcholine may lie hidden in such extracts as a physiologically inactive complex which is broken down by trichloroacetic acid treatment. In my first Freiburg experiment, however, this hypothetical complex apparently survived that stage, and only after precipitation with Reinecke acid did normal, physiologically active acetylcholine appear. Since a fundamental disagreement still exists between Kapfhammer's results and our own, it is superfluous to discuss this hypothetical complex. Apart from the chemical difficulties which it presents, its inconstancy in appearance and behaviour is perplexing; and in our investigations at Hampstead we have never encountered it.

Returning from Freiburg to Hampstead, I again examined ox blood from four different animals, using the technique with which I had become familiar in Kapfhammer's laboratory. In none of the extracts was more than a trace of acetylcholine-like activity (equivalent to not more than 0·1 mg. of acetylcholine chloride per litre of blood) physiologically detectable at any stage in the fractionation, which in each experiment was carried to completion in spite of the apparent absence of acetylcholine. The gold salts finally obtained consisted mainly of choline chloroaurate.

In order to test my technique, the unconcentrated alcoholic extract of the fourth sample (2 litres) of blood was divided into two equal quantities, to one of which I added 40 mg. of acetylcholine chloride. The two portions were then worked up simultaneously. Without difficulty, and with relatively little loss, the added acetylcholine was recovered, and finally identified as chloroaurate.

For the complete failure of the experiments made in this laboratory to confirm the positive results of those performed under precisely the same technique in Freiburg, we can find no reasonable explanation.

It should be mentioned that, since this work was completed, a short note by Vogelfanger [1933] has appeared, stating that there is no difficulty in obtaining from ox blood, by Kapfhammer's method, acetylcholine in amounts similar to those which he obtained. The author notes that the amount of oxalic acid recommended by Kapfhammer is inadequate to ensure a proper acidity of the initial extract, and emphasizes the necessity of adjusting the amount to that end. It might be implied that the failure of others to find acetylcholine in ox blood was attributable to neglect of this point. This criticism does not apply to our earlier work, for we ourselves discovered this defect in Kapfhammer's original directions, when applied to English ox blood, and took the

necessary steps to correct it [Dale and Dudley, 1931]. Apart from this critical note on a point already recognized and dealt with by us, Vogelfanger's account is a bare record, and does not assist to an understanding of our negative findings. Our numerous and consistently negative results prove that acetylcholine cannot be an artefact, and the erratic appearance of the substance in physiologically detectable form at different stages of the fractionation in different experiments rules out the improbable suspicion that there might be occasional batches of alcohol current in Germany which were contaminated with acetylcholine.

Since the same technique gives me positive results, confirming Kapfhammer's, in Freiburg, and negative results, confirming all our earlier observations, in London, it is obviously desirable that the question should be tested by others as widely as possible. Already Wrede and Keil [1931] have described negative results and Vogelfanger positive.

I earnestly hope that the publication of this paper will stimulate others, in this country and abroad, to investigate the problem. The method of Kapfhammer and Bischoff is admirably suited to its purpose, and has transformed the process of isolation of acetylcholine from one of great labour and difficulty to one of ease and simplicity.

In the following paper Chang and Gaddum publish experiments made on extracts of other tissues by physiological methods. Only in one case, that of the human placenta, as earlier in the case of horse and ox spleen, do these further experiments detect quantities of acetylcholine comparable to those found by the Freiburg investigators. With all other tissues, the experience of this laboratory entirely fails to confirm the presence of still higher proportions (nearly 200 mg. per kg. in some cases) of acetylcholine found by Kapfhammer and his collaborators.

Experimental.

The method of Kapfhammer and Bischoff, employed in the experiments under discussion, was carried out in the following manner in Freiburg.

Ox blood was collected at the municipal slaughter-house, where oxalic acid (1·6 g. per litre of blood) in saturated aqueous solution was added. It was then brought to the laboratory, a journey of about 20 min. duration, where 1 litre was poured with stirring into 5 litres of 96 p.c. alcohol. The reaction of the mixture was tested with blue litmus paper. As no definite acid reaction was detectable, aqueous lactic acid was added until the solution produced a faint reddening of litmus.

After standing for 1½–2 hours the solution was filtered through paper and mixed with the liquid expressed in a tincture press from the blood coagulum. The blood cake was re-extracted with 2 litres of 96 p.c. alcohol, which, after 2 hours' contact, was filtered off. The combined extract was then evaporated *in vacuo* (water bath at 40°) to 100–150 c.c.

An equal volume of 20 p.c. trichloroacetic acid was added to this concentrate and the mixture was placed in an ice-chest (4°) for 2 hours. It was then shaken out with ether four times, and the solution, now faintly acid to Congo-red, was concentrated *in vacuo* to approximately 100 c.c. Saturated aqueous Reinecke acid was added in slight excess and the mixture was placed in the ice-chest for 12–16 hours.

The precipitate was then filtered off and washed with ice-cold water until the runnings were practically colourless. Ice-cold alcohol was then applied; the runnings were at first faintly coloured, but speedily became colourless. After a final washing with ether the residual precipitate was dried in a desiccator. It was then dissolved in 25–50 c.c. of dry acetone. The solution, after removal of a small amount of insoluble material by filtration, was mixed with dry benzene (10–15 vols.). This precipitated the "reineckate" quantitatively, and it was collected on a folded filter paper. After drying, it was dissolved in about 100 c.c. of 50 p.c. acetone, excess of aqueous silver sulphate solution was added, the precipitate of silver "reineckate" removed in the centrifuge and to the liquid a solution of barium chloride, equivalent to the silver sulphate used, was added. The solution, freed from silver chloride and barium sulphate in the centrifuge, was concentrated *in vacuo* to 5 c.c. A strong solution of gold chloride, neutralized to Congo-red with sodium hydrogen carbonate, was added to this solution. After standing in the ice-chest for about an hour the gold salt was filtered off and recrystallized from water.

The experiments made at Hampstead were carried out strictly in accordance with the technique just described. The amount of lactic acid added to each alcoholic filtrate was 1 c.c.

The "reineckate" was precipitated from acetone solution by means of benzene for the sake of strict conformity, but this step does not appear to serve any useful purpose and could be omitted.

Table I shows the amounts of acetylcholine determined physiologically at each stage of the fractionation of the two Freiburg specimens and of the fourth sample of English ox blood, which was worked up in two equal

TABLE I. Acetylcholine in ox blood (mg. acetylcholine chloride per litre).

	Freiburg experiments		Hampstead experiments	
	Blood I	Blood II	Blood IV a	Blood IV b
Alcoholic extract before conc.	—	22 (approx.)*†	—	—
Alcoholic extract after conc.	—	—	0·13†	40†
After trichloroacetic acid purification	0·1–0·5*	27†	0·13†	40†
After Reinecke acid precipitation	40†	25†	0·09† 0·05‡	33·3† 34·4‡
Weight of chloroaurate after recrystallization	50 mg. (M.pt. 162°; Au. 40·4 p.c.)	30 mg. (M.pt. 163°; Au. 40·4 p.c.)	40 mg. (M.pt. 258°; Au. 44·3 p.c.)	54 mg. (M.pt. 164°; Au. 40·5 p.c.)

Physiological determination: * Cat's blood-pressure; † Rabbit's intestine; ‡ Frog's eserinized rectus. Au. calc. for acetylcholine chloroaurate, 40·6 p.c.; for choline chloroaurate, 44·5 p.c.

portions, to one of which (IV b) 40 mg. of acetylcholine chloride had been added. The other three English ox bloods gave results similar to those of IV a. When such small amounts of acetylcholine as appear in blood IV a are under examination the frog's eserinized rectus gives a much more accurate assay than does the rabbit's intestine. With the latter a fraction of the total activity still persists after atropinization of the test tissue. The amounts of twice recrystallized chloroaurate, with melting points and analyses, are also presented. The crude chloroaurate obtained from blood extracts always contains the choline salt. When the acetylcholine salt is also present the volume of water used for recrystallization is such that the choline salt remains in the mother liquor, since acetylcholine chloroaurate is much less soluble than choline chloroaurate.

It is to be noted that the exhaustive washing of the "reineckate" results in a small loss (15 p.c.) of acetylcholine.

Summary.

Kapfhammer and his colleagues in Freiburg maintain that ox blood contains relatively large amounts of acetylcholine, whilst we at Hampstead have consistently failed to confirm their finding.

This fundamental discrepancy persists in spite of a collaborative attempt, described in the present communication, to discover a reason for our disagreement.

In conclusion I wish to record my appreciation of the friendliness with which Prof. Kapfhammer received me in his laboratory, and of the expert and willing advice and assistance afforded me by Dr Bischoff in making my experiments there.

I am indebted to Sir Henry Dale, Dr J. H. Gaddum and Dr H. C. Chang for their help in making the necessary physiological determinations.

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CHOLINE ESTERS IN TISSUE EXTRACTS.

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Since Loewi [1921–30] brought evidence to show that the vagus nerve inhibits the frog's heart by liberating a substance pharmacologically identified as a choline ester, similar evidence has been obtained in experiments with a number of other nerves. It has been shown that the same mechanism is probably responsible for the action of the third nerve on the eye [Engelhart, 1931; Velhagen, 1931, 1932] of the "antidromic" vasodilator nerves to voluntary muscle [Dale and Gaddum, 1930; Feldberg, 1932; Bain, 1932], of the chorda tympani on the secretory cells of the submaxillary gland [Babkin, Stavraky and Alley, 1932; Babkin, Gibbs and Wolff, 1932; Gibbs and Szelöczey, 1932 *a*, *b*; Henderson and Roepke, 1932], and of the splanchnic nerve on the suprarenal medulla [quoted from Feldberg, 1933]. There is also some reason to believe that the intestine is capable of esterifying choline in the presence of the appropriate anions to form highly active choline esters [le Heux, 1919; Magnus, 1930], and that this mechanism is under control of the vagus [Hoet, 1925]. Fuller reference to work on this subject will be found in papers by Riesser [1931], Hirschberg [1931] and Kroetz [1931].

In 1925 Witanowski showed that a substance with the pharmacological properties of a choline ester could be obtained from frog's heart by mincing the tissue in alcohol. This observation has frequently been confirmed and similar results have been obtained with other tissues.

The only choline ester which has hitherto been isolated in a chemically pure state from animal tissues is acetylcholine, which was found to be present in large quantities in the spleens of horses and oxen [Dale and Dudley, 1929, 1931]. More recently Bischoff, Kapfhammer and

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their co-workers [1930, 1931, 1932] (see also Vogelfanger [1932]) have announced the isolation of even larger quantities of acetylcholine from blood, skeletal muscle, and many other tissues. Repeated attempts have been made in this laboratory to confirm these observations regarding blood and skeletal muscle and these attempts have been unsuccessful. We have, however, developed improved methods of making tissue extracts and testing them for acetylcholine.

In the discussion of our results we have found it convenient to use two new terms. The term "A.C.-substance" means "a substance pharmacologically identified as acetylcholine." "A.C.-equivalent" means "the apparent concentration of acetylcholine in terms of the chloride."

The preparation of extracts.

The conditions necessary for obtaining acetylcholine in extracts of horse's spleen have been discussed by Dale and Dudley [1929]. The spleen may be removed from the horse and kept intact for some hours without appreciable loss of acetylcholine, but as soon as the tissue is cut up or minced for extraction the acetylcholine rapidly disappears, and appreciable loss may occur even if the tissue is allowed to drop directly from the mincer into alcohol. In order to obtain maximum yields it is therefore important that the tissue should be soaked in some liquid which will inhibit the disappearance of activity, and disintegrated in this liquid.

The liquid which has most frequently been used in the preparation of such extracts is ethyl alcohol. Since it is known that acetylcholine is most stable in weak acids (*p*H 3·9, Hofmann, 1930) some workers add 1 c.c. of normal hydrochloric acid to each 100 c.c. of alcohol. We have obtained more satisfactory results with sulphuric acid, since extracts containing this acid filter more readily than extracts containing hydrochloric acid. In an experiment with dog's liver it was found that the final solution was of about the right acidity when each 100 g. of tissue was extracted with 400 c.c. of alcohol containing 5 c.c. of $2N$ H_2SO_4. A similar extract was then prepared from a horse's spleen and compared for activity on the rabbit's intestine with an extract made without the addition of acid. In this way it was found that the yield of acetylcholine was slightly increased by the addition of this quantity of sulphuric acid.

Trichloroacetic acid was used by Galehr and Plattner [1928] to precipitate the proteins in blood and thus stop the destruction of acetylcholine. In three experiments we have found that extracts of horse's spleen and intestine prepared by means of this acid contained 12–30 p.c.

more activity than similar extracts prepared from the same tissue with acid alcohol as described above, and we have therefore adopted the following method of preparing extracts:

> The tissue is weighed and placed in 10 p.c. trichloroacetic acid (2 c.c. of acid for each g. of tissue). It is cut up in the acid with scissors and then left with occasional stirring for 1–2 hours. The extract is filtered through paper on a Buchner filter and the tissue washed with 7 p.c. trichloroacetic acid. The extract is then shaken four or five times with ether in a separating funnel until it is only faintly acid to congo. The extract is usually left at this stage in the cold room overnight. It is then concentrated at low pressure at about 40° until 1 c.c. of extract corresponds to 1–10 g. of tissue. This solution is made neutral to congo-paper and then used in the biological test. It was found that cooling the acid and tissue to 5° before disintegration resulted in no further increase in the yield, and the extracts have therefore been prepared at room temperature.

It is known that the esterase which destroys acetylcholine is inhibited by eserine, and it is therefore possible that, if eserine were injected into an animal before it was killed, larger yields of acetylcholine would be obtained from its tissues. However, it is improbable that this would be so when the tissue is disintegrated beneath the surface of a solution of trichloroacetic acid. The acetylcholine in a horse's spleen appears to be stable until the tissue is disintegrated, and very little destruction can occur in the fraction of a second which elapses after a piece of tissue is cut and before the enzyme is inhibited by the trichloroacetic acid. We have not adopted previous eserinization as a routine procedure because it was desirable that the extracts of tissues should be free from traces of eserine. On the one occasion on which an extract was made from the submaxillary gland of an eserinized dog, the yield of acetylcholine activity was below the average of all the results obtained with the submaxillary glands of normal dogs.

Extracts prepared in the way described above contain large quantities of other pharmacologically active substances besides acetylcholine, and are only suitable for use in a biological test when the physiological reaction used in this test is one which is relatively little affected by such substances as potassium, histamine and adenosine compounds. For experiments with the frog's heart it is necessary to purify the extracts further, and the methods which are used for this purpose may lead to the partial disappearance of acetylcholine. Thus, in recent papers, Plattner and Krannich [1932] describe experiments in which they used the frog's heart to test quantitatively for acetylcholine in extracts of frog's voluntary muscle. The tissue was extracted with acid alcohol and the extract was taken down to dryness on a water bath. The residue was then shaken with absolute alcohol and again filtered. This process was repeated

several times and the final residue was taken up in Ringer's solution and tested. It seemed to us to be unlikely that acetylcholine would survive repeated evaporation to dryness on a water bath, and we have carried out an experiment to test this point. For this purpose we tested extracts of horse's spleen in comparison with acetylcholine, by means of the rabbit's isolated intestine. The experiment was carried out in this way, because we know with certainty that the effect of this extract on this tissue is mostly due to acetylcholine. Two extracts were prepared—one by Plattner and Krannich's method, and the other by the method using trichloroacetic acid, described above. Both extracts contained a substance which stimulated the intestine and which was easily destroyed by alkali, but the yield obtained by Plattner's method was only about 1/30 of that obtained by the other method. We concluded that 97 p.c. of the acetylcholine originally present in the tissue was lost in carrying out Plattner and Krannich's method of purification. It is not, of course, possible to conclude from this that similar losses would occur when the same method of extraction is applied to extracts of voluntary muscle, and we therefore do not wish to suggest that the active principle studied by Plattner and Krannich was not acetylcholine. Indeed, they present evidence that no other substance hitherto isolated from animal tissues could be responsible for the effects which they observed. Nevertheless, we do not think that this method of extraction can give a reliable estimate of the original acetylcholine content of tissues.

The biological testing of extracts.

A large number of different pharmacological preparations are affected by small doses of acetylcholine, and many of these are suitable for testing extracts of horse's spleen, which contains so much acetylcholine that the presence of other substances does not affect the result. However, the extracts which we have prepared from other tissues did not contain these large quantities of acetylcholine, and some of the methods which we have tried have proved unsuitable because the effect of tissue extracts on them was largely due to substances other than acetylcholine. The difficulties encountered in using some of these preparations are discussed below.

The frog's heart.

Straub's preparation of the frog's heart has been used by various investigators in testing extracts for acetylcholine. If a constant volume (say 0·1 c.c.) of different dilutions of the extract is added to the solution

(*ca.* 1 c.c.) in the cannula until a concentration is found which produces a definite decrease in the size of the beat, extracts can be compared quantitatively with reasonable accuracy (10–20 p.c.) by giving the two solutions in alternate doses. Different hearts vary rather widely in their sensitivity, but it is usually possible by this method to detect doses as small as 0·01 γ of acetylcholine, or less. The frog's heart is also affected by a very large number of other substances which are known to be present in tissue extracts. Small quantities of acid produce an effect similar to that of acetylcholine, so that extracts must be carefully neutralized before they are tested. This introduces a further complication, since acetylcholine is unstable unless the solution is kept slightly acid. Potassium (0·25 mg. KCl) produces an effect very similar to that of acetylcholine. An increase in the size of the beat may be produced by calcium or histamine, or by some substance associated with various complex fats [Clark, 1913; Eggleton, 1926], and the presence of these substances might mask the presence of acetylcholine. Adenosine, adenylic acid and adenylpyrophosphate appear to have no effect on the size of the beat, but they slow the heart and may stop it altogether if they are not removed from extracts.

Effects due to choline and its esters can be distinguished from effects due to any other substance known to occur in the body by the fact that they are antagonized by atropine. We have not found this test to be always easy to apply, because the sensitivity of the preparation to acetylcholine often decreases during the course of an experiment, even in the absence of atropine, when the preparation is treated with various tissue extracts. In any case it is impossible to carry out an assay of an extract for choline esters in the presence of significant quantities of the other substances mentioned, and this fact has led to the use of rather drastic chemical procedures for the removal of these undesirable substances. We have already discussed the probability that some of the procedures which have been used may also remove a large proportion of the acetylcholine when it is present.

Unknown substances may also interfere with the use of the frog's heart in testing for acetylcholine. Evidence for this statement is shown in Fig. 1 which shows the effect of an extract of rabbit's voluntary muscle which had been subjected to several stages of purification. The original extract had been made with trichloroacetic acid. This had been shaken with ether, and then taken down to a small volume. Nine volumes of alcohol were then added and the precipitate filtered off. The alcohol was removed from the filtrate, which was then treated with Reinecke

acid to remove choline and other bases. The filtrate was treated with silver sulphate to precipitate the excess of Reinecke acid, and then with $BaCl_2$ to precipitate silver sulphate. Lastly an excess of sodium sulphate had been added to remove barium, and the solution was neutralized to litmus. The tracing (Fig. 1) shows that it contained a substance which inhibited the frog's heart in much the same way as acetylcholine though its action persisted when the heart was rendered insensitive to acetylcholine by means of atropine. The solution was also tested by means of the frog's rectus as described below, and it was found to contain no detectable acetylcholine. A blank control (B.C.) was carried out which

Fig. 1. Frog's heart (Straub's method). *M.* Purified extract of voluntary muscle (0·25 g. tissue). *A.M.* The same after ashing. *B.C.* Blank control (reagents alone). *A.C.* Acetylcholine (0·05γ).

showed that the active substance was not derived from the reagents. We have at present practically no evidence as to the nature of the substance responsible for this effect, which we refer to as substance *R*. It is shown in Fig. 1 that substance *R* is destroyed by ashing and is therefore not potassium. When an extract was boiled for 5 min. with an equal quantity of 2*N* NaOH substance *R* was partially, but not completely, destroyed. When the extract was similarly treated with HCl a smaller proportion of the activity was destroyed. When an extract was taken to dryness and shaken with absolute alcohol substance *R* was detected in the alcohol.

The properties of substance *R* are such that we are unable at present to devise any simple method of removing it from extracts and leaving the acetylcholine intact. The procedures used by Plattner and Krannich

[1932], and discussed above, would be likely to preserve substance *R*, and we do not know why this substance did not inhibit the frog's heart, in their experiments, after atropinization. If this substance is present in frog's muscles in as high concentration as in rabbit's muscle it would certainly be expected to do so. Plattner and Krannich found that the effect of their extracts was abolished by atropine, that the active substance was destroyed by blood, and that acetylation did not increase the activity to such a large extent as that of choline is increased. Acetylcholine is the only substance which is known to occur in animal tissues, and to have this combination of properties, and there is no reason to doubt that the effects observed by Plattner and Krannich were, in fact, due to acetylcholine. We have not investigated this point more closely, because we believe that the evidence already obtained clearly indicates that the methods used by Plattner and Krannich give a less reliable indication of the acetylcholine content of animal tissues than other methods described later in this paper.

Rabbit's intestine and rabbit's blood-pressure.

These two preparations, unlike the corresponding preparations prepared from cats, are very insensitive to histamine, and we have therefore tried to use them as test objects for acetylcholine. They have been found unsuitable owing to the difficulty of excluding the complicating effects of two groups of substances:

(1) Adenosine and its derivatives which inhibit the intestine and depress the blood pressure. These substances can, however, be almost completely removed by taking advantage of the fact that they are insoluble in high concentrations of alcohol.

(2) An unknown substance which we call substance *P*—some of whose properties have already been described [Euler and Gaddum, 1931]. This substance depresses the blood-pressure and stimulates the intestine so that in these respects it simulates acetylcholine, but its effects are not abolished by atropine.

Cat's denervated gastrocnemius.

About a week after preliminary aseptic section of the sciatic nerve the cat's gastrocnemius becomes sensitive to acetylcholine and other drugs allied to nicotine [Frank, Nothmann and Hirsch-Kauffmann, 1922; Dale and Gasser, 1926]. This phenomenon may be used as the basis of a sensitive and specific test for acetylcholine in tissue extracts.

The following are the details of the technique which we have found to be most satisfactory.

The preliminary, aseptic nerve section is performed under ether anæsthesia a week before the experiment. The cat is then anæsthetized with chloralose and artificial respiration is applied. The last lumbar arteries and all lower branches of the aorta except the two external iliac arteries are tied. The denervated gastrocnemius is freed, and the femoral artery is tied immediately below it. The tension is recorded isometrically and the optimum initial tension is determined at the beginning of each experiment. Warm Ringer's solution is made to flow continuously over the muscle. Eserine (0·2 mg.) is injected intravenously to increase the sensitivity, and atropine (5 mg.) is injected subcutaneously to abolish the vasomotor effects of acetylcholine, which are liable to cause variations in the response. Atropine does not affect the reaction under study. Injections are made into the base of the aorta through the central end of the external iliac artery running to the normal leg by means of the special device illustrated in Fig. 2. This consists of a small cannula into which is inserted a rubber cap, slit at top so as to act as a valve. In the figure a brass fitting, made from one of the needles supplied with the syringe, is shown inside the rubber cap. This was found to be a convenient addition to the apparatus, but in most of the experiments injections were made by fitting the nozzle of the syringe directly into the rubber cap. The dead space between the valve and the artery is only about 0·05 c.c., and this is negligible if the volume of the injection is 0·5 c.c. or more. Small quantities of active substances which might remain in this space are washed out by injecting saline in between each main injection. Blood is rather liable to enter the mouth of the cannula between injections and clot there. This tendency can be largely avoided by using cannulæ whose mouths are reduced to a hole so small as to be scarcely visible, but consistently satisfactory results have only been obtained when the cat has received a preliminary injection of heparine. The main advantages of this type of cannula are that none of the injected solution is wasted except the quantity required to fill the inside of the rubber cap (about 0·1 c.c.) and that, if the cannula is fixed in a clamp, injections can be made without mechanically disturbing the cat. Injections can be made at intervals of about 5 min. In satisfactory preparations doses of 0·1γ or 0·2γ of acetylcholine cause consistent reactions under these conditions.

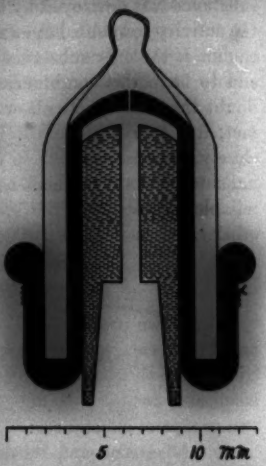

Fig. 2. Glass cannula for intra-arterial injections, containing rubber cap to act as valve, and brass fittings to receive nozzle of syringe.

The result of a comparison carried out in this way is shown in Fig. 3. The continuous straight line in this figure shows the effect that would presumably have been produced by 0·24γ of acetylcholine. It will be seen that 0·6 c.c. of the test solution had less than this effect and 0·9 c.c.

had more. The preparation is fairly sensitive and appears to be at least as specific as any other known test for acetylcholine. It is presumably unaffected by the presence of atropine in the extract tested. Its chief disadvantage lies in the fact that it is excessively laborious.

Fig. 3. Cat. Atropine and Eserine. Tension of denervated gastrocnemius. Unknown solution, *T*, compared with a constant dose of acetylcholine.

Frog's rectus abdominis and leech muscle.

The frog's rectus abdominis and the longitudinal muscle of the leech (*Hirudo medicinalis*) both provide sensitive and specific test objects for acetylcholine in tissue extracts. In both cases the preparation is specifically sensitized to acetylcholine by treatment with eserine, so that this drug may be used to distinguish acetylcholine from other substances which might be present in extracts.

The action of acetylcholine on the frog's rectus was first described by Riesser [1921] and has since been widely studied, but has, apparently, not been used in the testing of tissue extracts. The muscle obtained from *Rana esculenta* is rather more sensitive than that obtained from *R. temporaria*, but either species can be used.

The action of acetylcholine on the eserinized leech was first described by Führer [1918*a*, *b*]. The use of this preparation for the assay of extracts for acetylcholine was recommended by Minz [1932] while the present work was in progress. The reaction is unaffected by atropine and abolished by nicotine. In our experience the leech has not proved such a satis-

factory test object as the rectus, but it may be used with advantage to confirm the results obtained with the rectus, and as the two preparations have much in common it is convenient to describe them together.

The bath we have used for both preparations contains 18 c.c. of Ringer's solution at room temperature. The magnification was about 10 and the tension about 3 g. wt. for the rectus, and more for the leech. The doses used were only slightly greater than the threshold dose. In both cases the action of acetylcholine is a very slow one, and we have found that two solutions of acetylcholine can be satisfactorily compared in a relatively short time by means of the following device. The drug dissolved in a volume of less than 0·5 c.c. is added to the bath and left in contact with the preparation for exactly 3 min. (or, with some preparations, exactly 2 min.). After this time the effect is usually not quite complete, but, nevertheless, the solution is changed and the preparation allowed to relax. If doses are added at a constant time interval of 10 min. the effect produced in the given constant time is regularly related to the dose, and can be taken as an index of the potency of the solution. When a small effect has been obtained with the extract on the normal preparation, 0·2 mg. of eserine sulphate is added to the bath so as to give a concentration of eserine of about 10^{-5}. This concentration is more than sufficient to give a nearly maximal sensitization to acetylcholine, but this sensitization takes about half an hour to develop. If the extract contains detectable quantities of acetylcholine its action is then very definitely increased, and a quantitative estimate of the acetylcholine equivalent of the extract is obtained by comparing it with a solution of pure acetylcholine given in alternate doses. 0·2 mg. of eserine sulphate is always added to the bath after the solution has been changed. This is more satisfactory than adding the eserine to the main bulk of Ringer solution, because eserine is not very stable in high dilutions, except in the presence of acid.

Fig. 4 shows the action of a series of substances on the frog's rectus. 12 mg. of KCl, 2γ of acetylcholine and 2 mg. of choline produced, in this experiment, effects of roughly equal size, but of very different shapes. These shapes are characteristic of these three substances. Potassium causes an initial twitch followed by a slow contracture, and acetylcholine differs from choline in that its effect starts more quickly and describes a curve which is more convex upwards. The last three effects were obtained after eserine had been added to the bath. The actions of choline and potassium were practically unchanged, while that of acetylcholine was increased.

The leech preparation differs from the rectus preparation in the following aspects:

(1) In our experience it was usually difficult to obtain a satisfactory

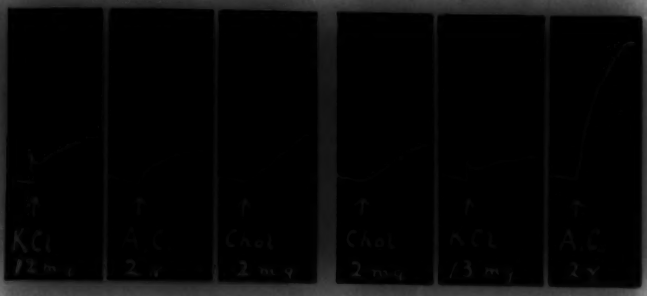

Fig. 4. Frog's rectus abdominis in 18 c.c. bath. First three doses—no eserine. Last three doses 10^{-4} eserine sulphate.

Fig. 5. Frog's rectus abdominis. Eserine sulphate 10^{-3}. *R.* Extract of dog's right submaxillary gland. Dose in g. of tissue. *A.C.* Acetylcholine.

comparison with the leech because of wide variations in the length to which the preparation relaxed when the drug was washed out.

(2) Before eserine, acetylcholine has no effect on the leech, except in very large doses. After eserine the leech is slightly more sensitive than

the rectus. A specially reactive rectus is then about as sensitive as an unusually insensitive leech. The effect of eserine on the leech is thus very much greater than its effect on the rectus.

(3) Potassium causes a contraction of the rectus, but a relaxation of the leech. The smallest dose which produces these effects is about the same in the two cases. There is thus no danger of confusing the effect of

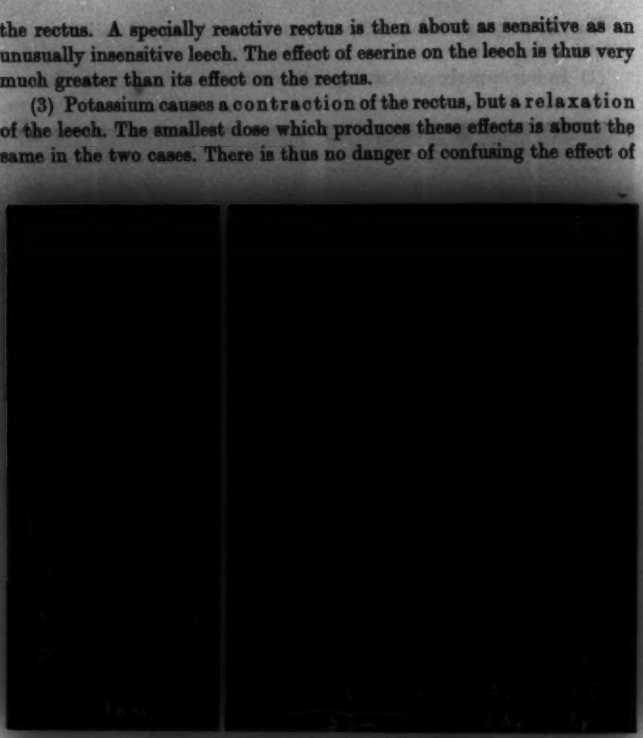

Fig. 6. Frog's rectus abdominis. Eserine sulphate (10^{-6}) added between the two sections of tracing. Extracts of dog's pancreas, lung and spleen. Dose in g. of tissue. *A.C.* Acetylcholine.

potassium on the leech with that of acetylcholine, but a large amount of potassium may mask the presence of a small amount of acetylcholine. In some cases where the suspicion has arisen that effects observed on the rectus were due to potassium, the suspicion has been confirmed by testing the extract on the leech.

Histamine, adenosine, calcium, and the substances *P* and *R* discussed above, given in large doses, had no action on either preparation.

Fig. 5 shows a comparison carried out with an eserinized rectus. *R* represents an extract of a dog's right submaxillary gland. The plateaux at the maxima of the effects represent the times during which the lever was arrested and the solution changed. In this experiment the volume of extract corresponding to 0·17 g. of gland was equivalent to something between 0·25γ and 0·21γ of acetylcholine.

Fig. 6 shows the result of some tests near the limits of the sensitivity of the method. The first three doses correspond to 3 g. of a dog's pancreas, lung and spleen. The small effects obtained were probably due to potassium. The preparations were now eserinized and it was found that the pancreas and lung contained some detectable acetylcholine-like substance, but the spleen did not. It was subsequently found that if the spleen had contained as much as 0·1γ of acetylcholine per g. this quantity would have been detected in this test.

The pharmacological differentiation of acetylcholine from other substances.

The following special tests have been used to differentiate between effects due to acetylcholine and those due to other substances.

(1) If the action of an extract is increased by eserine this fact is in itself sufficient to justify the conclusion that the effect is not due to choline—or to any other substance, except acetylcholine, which is known to be present in tissues. This is certainly true for tests on the frog's rectus and the leech, but eserine cannot always be used in this way when other pharmacological preparations are used. For example, it has been found impossible to apply sufficient eserine to potentiate a rabbit's isolated intestine without producing a contraction due to the eserine itself, or, more probably, to potentiation of the action of a choline ester naturally present [Matthes, 1930].

(2) The activity should disappear rapidly when the extract is mixed with blood, but if the blood has been previously treated with eserine this reaction should be very greatly slowed. In applying this test it is sometimes found that the rate of destruction is different from the rate of destruction of pure acetylcholine under the same conditions. This observation does not in itself prove that the original effect was not due to acetylcholine, because it is possible that the rate of destruction is affected by other substances, such as choline, which may be present in the extract.

(3) The active principle should be unstable in alkaline solution. If a portion of an extract is mixed with an equal volume of 2*N* NaOH, kept

for 10 min. at room temperature, and then neutralized, any acetylcholine which it contains is destroyed, while choline is unaffected.

(4) The choline content of the extract may be determined by acetylating it and then comparing it pharmacologically with acetylcholine. It is possible to calculate, from the result of this test, whether the amount of choline originally present was sufficient to account for the action originally observed. Calculations of this type give no information about the active substance under study, except that it is not choline—a fact which can usually be proved by other simpler tests.

(5) Many of the effects of acetylcholine are antagonized by atropine. Other effects are antagonized by nicotine. The extract should react to these drugs in the same way as acetylcholine itself. Tests of this kind have served to differentiate effects due to histamine, adenosine compounds, potassium, and the substances *P* and *R* discussed above.

(6) When the activity of the extract is estimated quantitatively in terms of acetylcholine, using several different pharmacological tests, the same result should be obtained in each case.

The last of these methods probably provides much stronger evidence for the pharmacological identification than any of the others. For example, it serves to differentiate acetylcholine from other choline esters. The fact that the different kinds of activity which these substances possess may vary independently is well known (see, for example, Simonart, 1932), but in order further to illustrate the point we have compared several different esters with acetylcholine by five different methods. The results of these experiments are shown in Table I in which the apparent activity of each ester is expressed as a percentage of that of an equimolecular quantity of acetylcholine. Each of these figures represents a comparison in which the substance tested and acetylcholine were given in alternate doses until doses were found which produced equivalent effects. We are indebted to Dr H. W. Dudley for specimens of most of these esters, and to Messrs Merck for the specimen of carbaminoylcholine.

Sometimes the effects produced could not be satisfactorily compared, because the two substances described curves of quite different shapes on the drum. The figure obtained in such cases depends on the length of time the drugs are left in contact with the tissue, but usually the ratio measured in this way was approximately constant in any one experiment. In some cases the figure obtained was confirmed in a second experiment, but the exact result probably varies with uncontrolled alterations in the conditions of the experiment. Matthes [1930] found that the activity

of pyruvylcholine on the rabbit's intestine was 23 p.c. of that of acetylcholine. In our experiments the figure was 14 p.c. Variations of this kind are probably inevitable, and the figures given in Table I cannot be expected to be always accurately true. These considerations do not detract from, but tend rather to enhance, the value of parallel quantitative tests, as a means of differentiating between acetylcholine and other esters.

All of the esters given in Table I, except carbaminoylcholine, are active unstable substances whose effects on the rectus are increased by eserine to about the same extent as those of acetylcholine, and the first five of the special tests given above would probably not differentiate

TABLE I. Showing the relative potency per molecule, of choline and some of its esters measured by different tests in comparison with acetylcholine, the potency of which is taken as 100 in each case.

	Rabbit		Frog's rectus abdominis		
Substance	Intestine	Blood-pressure (depressor effect)	Normal	After eserine	Leech (after eserine)
Choline	0·075	0·005	0·14	0·035	0·015
Propionylcholine	3·000	4·000	550·00	450·000	45·000
Butyrylcholine	0·240	0	90·00	115·000	90·000
Valerylcholine	0·200	0	25·00	30·000	0·900
Glycollylcholine	0·220	0·250	1·20	1·000	0·130
Pyruvylcholine	14·000	10·000	13·00	13·000	16·000
Carbaminoylcholine	80·000	15·000	18·00	5·000	12·000

them. If, however, an extract containing one of these substances was tested in several different ways, there would in most cases be no difficulty in showing that its activity was not due to acetylcholine. Pyruvylcholine is exceptional in that the ratio of its activity to that of acetylcholine appears to be fairly constant, and it would probably be difficult to differentiate it by biological tests.

We have not been able to obtain a satisfactory estimate of the activity of many of these substances on the normal leech, because the preparation is very insensitive until it has been treated with eserine, and the quantities of the esters available were limited. The preparation was sensitized by eserine to all these substances except choline. In most cases the sensitization was very marked, but in confirmation of Minz's results [1932] we found that carbaminoylcholine was quite active on the normal leech (about six times as active as acetylcholine), and its action was comparatively little increased by eserine. As Minz points out, these facts are probably due to the fact that carbaminoylcholine is not readily attacked by the esterase present in the tissue.

The potency of choline on the normal leech was found to be about a quarter of that of acetylcholine, so that this preparation would be useless in testing for acetylcholine in tissue extracts, which always contain a large excess of choline. When treated with eserine, on the other hand, the leech forms a valuable test object, which can be used in parallel with the frog's rectus to test crude extracts.

The results of some of our experiments on the rabbit's blood-pressure confirm some results obtained by Simonart [1932] in experiments on the cat's blood-pressure. Simonart found that propionylcholine was a less active vaso-dilator than acetylcholine, and that butyrylcholine was less active still, but after atropine, when all three substances produced pressor effects, the order of their activities was reversed. In our experiments on rabbits the contrast between the dilator activities of these three substances was in the same direction, but more marked. Butyrylcholine showed no real depressor action because the initial small fall in blood-pressure which it produced was immediately complicated by a secondary pressor action, even in the absence of atropine. Valerylcholine was very similar to butyrylcholine in these respects.

Our results with carbaminoylcholine on the rabbit's blood-pressure do not confirm those obtained by Kreitmair [1931] and Nöll [1932], using cats. Nöll injected carbaminoylcholine into a spinal cat whose blood-pressure was raised by the slow infusion of adrenaline, and found carbaminoylcholine to be about 1000 times as active as acetylcholine. In order to test the possibility that this discrepancy might be due to the use of a different technique, we have repeated Nöll's experiment, using a spinal cat and infusing adrenaline at about the same rate. In this experiment the activity of carbaminoylcholine was about 40 p.c. of that of acetylcholine. We are unable to explain the discrepancy. We have obtained similar results in several experiments. We have been in correspondence with Dr Kreitmair and, in spite of his friendly assistance, have been unable to find any explanation of the fact that we consistently find carbaminoylcholine to be less active than acetylcholine on the blood-pressure of the cat and rabbit, while he finds it to be more active.

The apparent acetylcholine-content (A.C.-equivalent) of some tissues.

Extracts have been made of a large number of tissues by the method described above, and these have been compared with known solutions of acetylcholine chloride by means of the eserinized frog's rectus. The

acetylcholine equivalents, or apparent contents of acetylcholine calculated as the chloride are shown in Table II.

TABLE II. A.C.-equivalents (apparent acetylcholine chloride contents) of some tissue extracts.

Tissue	Animal	A.C.-equivalent (γ per g. of tissue)
Spleen	Horse. Ox	4–30
Placenta	Human	28
Submaxillary gland	Dog	1·9, 1·25, 1·5, 3·3, 1·05, 3·3, 2·5, etc.
—	Cat	1·5
Small intestine muscle	Dog	1·8
Small intestine mucous membrane	Dog	1·7 } , 2·6
Small intestine	Rabbit	4, 2·8
—	Horse	2·0, 1·8
Large intestine	Dog	2·0
Sympathetic nerve	Horse	1·3, 3·3, 3·9
Auricle	Dog	1·2, 1·3, 0·93, 1·9
Bladder	Dog	1·2, 1·2
Stomach	Rabbit	1·1, 1·3
—	Dog	0·7
Parotid gland	Cat	0·9
Suprarenal gland	Horse	0·6, 0·4
Pancreas	Horse	0·6
Eye	Rabbit	0·6
Fœtus	Rabbit	0·45
Œsophagus	Cat	0·4
Uterus	Rabbit	0·4
Brain	Dog	0·4
Vagina	Dog	0·3
Ventricle	Dog	0·1, 0·07, 0·21, 0·35
Trachea	Dog	0·2
Pancreas	Dog	0·13, 0·22
Lung	Dog	0·13
Liver	Dog	0·11
Skin	Rabbit	0·1
Skeletal muscle	Horse	0·08, 0·08, 0·06
—	Rabbit	0·06
Blood	Horse	0·05, 0·065
—	Dog	0·05, 0·05
—	Ox	0·08, 0·08, 0·06
Plasma	Horse	Nil (<0·015)
Placenta	Rabbit	Nil (<0·1)
Fat	Dog	Nil (<0·1)
Testis	Dog	Nil (<0·1)
Kidney	Dog	Nil (<0·1)
Diaphragm	Dog	Nil (<0·1)
Mesentery	Dog	Nil (<0·1)
Spleen	Dog	Nil (<0·1)

In every case in which a figure is given for the A.C.-equivalent the effect was due to some substance, the action of which was increased by treating the preparation with eserine. In the case of those extracts in which no figure is given we can say with confidence that there cannot have been as much as 0·1γ of acetylcholine per g. of tissue. Quantities smaller than this cannot always be detected with certainty by this

method because it is necessary to use very highly concentrated extracts, and when crude concentrated extracts are made from some tissues they contain sufficient potassium to interfere with the test.

In some respects these results conform with expectation. The tissues with the highest A.C.-equivalent are mostly tissues whose main activity is controlled by nerves which may be supposed to act by liberating acetylcholine, but there are certain unexplained anomalies.

Blood.

A separate discussion is published [Dudley, 1933] of the attempts that have been made to account for the fact that the results obtained in this laboratory fail to confirm the observation of Kapfhammer and Bischoff [1930], that normal blood may contain large quantities of acetylcholine. By means of the frog's rectus it has been possible to detect an acetylcholine-like activity of a very low order, corresponding to a fraction of 1 p.c. of the quantities found by the Freiburg workers. Although the other tests used would have been sufficiently sensitive to detect quantities of acetylcholine considerably smaller than those stated to be sometimes present, they were less specific than the test on the frog's rectus and failed to detect any activity which could, with certainty, be attributed to acetylcholine. The following account is confined to a description of the results obtained with the frog's rectus.

In one experiment 200 c.c. of citrated jugular blood from a horse were centrifuged, and the plasma was poured off. A concentrated extract of this plasma was prepared by the method recommended above, using trichloroacetic acid. No activity could be detected in this extract, though the concentration of the solutions used was such that as little as 0·015γ of acetylcholine per c.c. of original blood would have produced an effect if it had been present.

A similar extract of the blood cells, also prepared by simple precipitation with trichloroacetic acid, appeared at first to contain about 0·4γ of A.C.-equivalent per c.c. of blood, but the rectus was not sensitized to this extract by eserine, and the effect was probably largely due to potassium. Twenty volumes of absolute alcohol were added to the extract, the precipitate was filtered off and the alcohol removed *in vacuo*. The contraction of the rectus which the extract now produced was increased in the presence of eserine, though to a rather smaller extent than that due to acetylcholine, and the activity was equivalent to 0·065γ of acetylcholine per c.c. of blood. Three extracts of ox blood prepared by Dr Dudley and purified according to Kapfhammer and Bischoff's

method by means of Reinecke salt were also tested on the frog's rectus and contained activity corresponding to about 0·09, 0·08 and 0·06γ acetylcholine per c.c. of blood. A record of the effect obtained in one of these experiments is shown in Fig. 7. It will be seen that, in this case also, the effect was increased by eserine, but not to the same extent as that of acetylcholine. This may be due either to the fact that the substance in blood responsible for these effects was not acetylcholine, or that it was acetylcholine mixed with sufficient choline, or other substances, to affect its action. The aurichlorides prepared from these

Fig. 7. Frog's rectus abdominis. Eserine sulphate (10^{-6}) added at 3.40 p.m. *B.* Purified blood extract (see text) concentrated 16 times. *A.C.* Acetylcholine.

extracts also showed activity of the same kind. These results are very different from those reported by Bischoff, Grab and Kapfhammer [1931] who found in some cases as much as 40γ of acetylcholine per c.c. of blood.

The small quantity of A.C.-equivalent which we were able to detect in blood was removed by centrifuging, and was therefore bound in some way with the blood cells. It has been shown that, after a dose of eserine, acetylcholine or some similar substance may be present in the blood in the submaxillary veins [Babkin, Stavraky and Alley, 1932], and in

the portal veins [Feldberg and Rosenfeld, 1933]. In both cases the active substance must have been in the plasma. The concentrations detected in these experiments were of the same order as the concentrations which we have been able to detect in normal blood by extraction with trichloroacetic acid and alcohol.

Voluntary muscle.

In the experiments of Bischoff, Grab and Kapfhammer [1932] an extract of voluntary muscle from an ox contained as much as 194γ of acetylcholine per g. of tissue. We have not tried, as in the experiments with blood, to imitate every detail of the experiments of these workers, but we have prepared ten extracts by different methods, suitable for the conservative extraction of acetylcholine if present, from the muscles of the horse, dog, cat, rabbit and frog, and have never obtained evidence of more than 0·08γ of A.C.-equivalent per g. of tissue. In this respect our results are similar to those of Plattner and Krannich [1932].

Spleen.

Dale and Dudley [1929] drew attention to the contrast between the large amount of acetylcholine contained in the spleens of horses and oxen, and its apparent absence from the spleens of dogs and other smaller animals. The reality of this contrast has now been confirmed by a more specific test. The extracts of dog's spleen did not contain as much as 0·1γ of A.C.-equivalent per g. of tissue.

Placenta.

We have been able to confirm the observation of Bischoff *et al.* [1932] that extracts of human placenta contain very large quantities of a substance pharmacologically identified as acetylcholine (28γ per g. of tissue). The extract was prepared with alcohol and compared with acetylcholine for its action on a piece of rabbit's intestine, and on the frog's rectus abdominis. The results obtained in these two comparisons agreed quantitatively with one another. On the other hand an extract of rabbit's placenta contained no detectable acetylcholine.

The presence of these large quantities of acetylcholine in the human placenta is as difficult to relate to any known physiological function as that of similar quantities in the spleens of large ungulates. In neither case is it yet possible to offer any explanation of the presence of a high concentration of acetylcholine, limited to certain organs of certain species. We have not discovered any similar anomalies among the other extracts we have tested.

Suprarenal gland.

The results of the tests of extracts of suprarenal gland are of rather special interest because the original investigation of the pharmacological properties of acetylcholine was undertaken by Hunt and Taveau [1909] because they had found that the vaso-dilator effects of extracts of suprarenal from which the adrenaline had been removed could not be entirely due to choline, and they believed that some simple derivative of choline might be partly responsible for the effects observed. Our conclusion that suprarenals contain a substance pharmacologically identified as acetylcholine confirms the result of Minz's experiments with leeches [1932].

Sympathetic nerves.

The observation that extracts of a horse's sympathetic nerve appeared to contain large quantities of acetylcholine was surprising. In one experiment the estimate obtained on a frog's rectus was quantitatively confirmed in an experiment with an eserinized leech. In two other experiments attempts were made to discover whether the activity was more concentrated in the ganglia, or in the parts of the sympathetic chain between the ganglia. The ganglia are not very distinct structures in the horse, but the portions of the nerve immediately surrounding the point of entrance of the rami communicantes were dissected out and extracted separately from the rest of the nerve. The estimates of the A.C.-equivalents of the two extracts prepared in this way were, however, identical.

It is known that acetylcholine has a marked action on sympathetic ganglia, and these results therefore suggested the theory that acetylcholine might play a part in the normal transmission of impulses through ganglia. We have obtained no direct evidence in favour of this theory, but the recent work of Kibjakow [1933] seems to support it. Kibjakow has shown that the stimulation of preganglionic fibres in the cat causes the liberation in the superior cervical ganglion of a substance capable of acting on ganglion cells. Our results suggest that this substance may be acetylcholine.

Heart.

The observation that extracts of mammalian auricles contain much larger quantities of A.C.-equivalent than extracts of ventricles confirms the conclusions of other investigators. Jendrassik [1923] found that watery extracts of rabbit's auricle stimulated an isolated rabbit's intestine while extracts of ventricle produced pure inhibition. Engelhart [1930],

working in conjunction with Plattner, found that the normal A.C.-equivalent of an alcoholic extract of rabbit's auricle, as measured on a frog's heart, was about 4γ per g., while that of the ventricle was only 0·13γ per g. These figures are of the same order as those recorded in Table II. This difference between the auricles and ventricles was foreseen by Engelhart on the grounds that the vagus has little or no direct action on the ventricles, but only affects them as a secondary consequence of its action on the auricles [see Dale, A. S., 1930].

Intestine.

The results obtained with extracts of intestine had also been foreshadowed. The intestine contains large quantities of choline. Choline has a powerful stimulant action on the isolated intestine, and acetylcholine has the same action in about one-thousandth of the concentration. Le Heux [1919] found that the salts of various acids which form active choline esters also stimulated the rabbit's intestine when added to the bath in which a piece of isolated intestine was suspended, and that this action was antagonized by atropine. He therefore suggested that the intestine was able to synthesize choline esters under these conditions. This conclusion was confirmed by much other evidence.

More recently Feldberg and Rosenfeld [1933] have found that the portal blood in an eserinized dog or monkey differs from systemic blood in containing a substance, pharmacologically identified as acetylcholine. This substance did not occur in the blood in the splenic vein and must have been derived from the intestine.

It was therefore probable that a choline ester would be found to occur naturally in extracts of intestine. Evidence on this point cannot be obtained by testing crude extracts, except when a highly specific pharmacological test such as that on the rectus or leech is used, because those extracts contain not only potassium, histamine and choline, but also adenosine compounds and the substance *P* [Euler and Gaddum, 1931].

In order to confirm the conclusions reached from the experiments with the rectus, an extract was therefore purified by chemical methods calculated to remove these other active substances. This extract was originally prepared for other purposes from horse's intestine without all the precautions that might have been applied to avoid partial hydrolysis of choline esters. An unknown loss therefore probably occurred before the first test was made, but the extract contained a substance unstable in alkali, and sufficient activity was present to enable the purification to be carried further. The original extract was in acid alcohol. This was taken

down *in vacuo* at 40° to small bulk and defatted with ether. A large quantity of absolute alcohol was then added and the precipitate, which was inactive on the frog's rectus, but which contained practically all of the substance acting like adenosine, was thrown away. The process was repeated until the extract could be taken down to dryness, and what was left of the activity (about 40 p.c.) was then dissolved in absolute alcohol. Platinum chloride was added. The filtrate from this contained no acetylcholine, and all that was left (about 22 p.c.) of the substance acting on the frog's rectus was in the precipitate.

The base was liberated from the chloroplatinate by means of metallic silver [Dudley, 1929], and was tested on the frog's rectus. The chloroplatinate was thus found to contain base corresponding in activity to 0·115 p.c. of acetylcholine chloroplatinate. The extract was acetylated, and again tested on the rabbit's intestine. From this result it would be calculated that the precipitate contained 8·4 p.c. of choline chloroplatinate, so that only about 1·4 p.c. of the total choline in the precipitate was apparently in the form of acetylester. The effect of the solution prepared from the platinum precipitate on the normal rabbit's intestine was indistinguishable from the effect of the amount of acetylcholine, which it had been estimated by means of the frog's rectus to contain, but when atropine was added, the extract, when given in large doses, still had an action—presumably due to the substance *P* [Euler and Gaddum, 1931]. This substance was almost completely removed by washing the platinum precipitate repeatedly with absolute alcohol—a procedure which reduced its weight by 27 p.c. without any detectable loss of the substance acting on the frog's rectus. The final precipitate was free of adenosine compounds and substance *P*, but presumably still contained histamine and large quantities of choline. It was tested on the rabbit's intestine and in the rabbit's blood-pressure and also by injection into the artery leading to a cat's gastrocnemius, which had been rendered sensitive to acetylcholine by denervation a fortnight before the experiment. The four estimates of the amount of acetylcholine in this precipitate are given in Table III.

The agreement between these results was considered satisfactory. The actions of this extract, like those of acetylcholine, on the rabbit's

TABLE III. Estimates of the A.C.-equivalent of a purified precipitate from horse's small intestine. Parts per thousand.

Frog's rectus	0·90
Cat's gastrocnemius	0·75
Rabbit's intestine	0·80
Rabbit's blood-pressure	0·84

intestine and blood-pressure were abolished by atropine, while that on the cat's gastrocnemius was not. The extract was treated with normal blood and with eserinized blood, and then deproteinized and tested on the frog's rectus and rabbit's intestine. Both types of test showed that the active substance was destroyed by blood and that the destruction was inhibited by eserine. This extract was not tested on the eserinized leech, or on the eserinized frog's rectus, because it was prepared at an early stage of the investigation before these simple tests were known; but, nevertheless, the experiments which were performed provide strong evidence for the identification of the substance in extracts of intestine, which is mainly responsible for the action on the frog's rectus, as acetylcholine. Pyruvylcholine is the only other substance in Table I which could have been responsible for these effects.

Other extracts.

Emphasis must be laid on the fact that in most other cases the only special test which was applied consisted in the observation that the action on the rectus was increased by eserine, so that the effect observed might have been produced by almost any choline ester. Indeed, the effects of extracts of dog's bladder differed sufficiently in their time relations from those of acetylcholine to suggest that they may really be due to some other choline ester. An extract of testis contained some substance which produced a contraction of the rectus similar to that caused by acetylcholine, and equivalent to 0·4γ of acetylcholine per g.; but as this effect was not increased by eserine it cannot have been due to acetylcholine, or to any choline ester likely to occur in a tissue extract, and this figure is not included in Table II. This phenomenon, which was observed twice, was not observed with extracts of other tissues.

The distribution of A.C.-equivalent shown in Table II is in some respects similar to the distribution of choline esterase shown by the experiments of Plattner and Hintner [1930]. Thus extracts of intestine, salivary glands, suprarenal, brain and uterus contained more than 0·3γ of A.C.-equivalent per g., and sufficient esterase to halve the concentration of acetylcholine in less than 10 min. under the conditions of experiment. Lung, ventricle, skeletal muscle and kidney contained less than these quantities of A.C.-equivalent and of esterase. On the other hand, blood and dog's pancreas, liver and spleen, failed to comply with this classification. These tissues were all rich in esterase and poor in A.C.-equivalent. There were no tissues rich in A.C.-equivalent and poor in esterase.

Factors affecting the A.C.-equivalent of tissues.

The most generally successful method of demonstrating the liberation of substances by nerves has consisted in stimulating the nerves to tissues bathed in salt solution, and then detecting the appearance of pharmacological activity in the salt solution. Experiments of this kind provide no evidence as to whether the active substance is actually formed in response to the stimulus or merely liberated from a pre-formed store. Evidence on this point can be obtained by measuring the A.C.-equivalent of tissue extracts before and after stimulation of the nerves. Experiments of this kind have been carried out on the heart. Witanowski [1925] showed that stimulation of the frog's vagus increased the A.C.-equivalent of the alcoholic extracts of the frog's heart. Plattner [1926] found that the same phenomenon could be demonstrated in the ventricles of rabbits, dogs and cats. Englehart [1930] confirmed these observations, but emphasized the fact that in mammals the auricles contain very much more A.C.-equivalent than the ventricles, and that the largest increase occurs in the auricles.

We have attempted to carry out similar experiments with the submaxillary glands, but have not succeeded in showing any marked and consistent change in the A.C.-equivalent of extracts when the chorda tympani was stimulated.

In these experiments the dogs were anæsthetized with ether followed by chloralose. The chorda (nervus submaxillaris) was stimulated on one side for 30–60 min. and 15–20 c.c. of saliva were secreted. The glands were then removed and immediately minced under the surface of 10 p.c. trichloroacetic acid. The extracts were treated as described above and tested on the eserinized frog's rectus. Within these limits the details of the procedure were varied in the different experiments in the hope that conditions would be found under which the stimulation would produce some recognizable change in the A.C.-equivalent of the gland. The results are recorded in Table IV. In the last of the experiments recorded in this table the dog had received an intravenous injection of 0·2 mg. of eserine

Table IV. Showing the A.C.-equivalent of dogs' submaxillary glands. Normal resting glands compared with those removed after stimulation of the chorda.

γ per g. of wet tissue		γ per gland	
Resting	Active	Resting	Active
4·0	4·0	20	19
1·3	1·5	11	12
1·2	1·1	8	10
2·5	2·5	14	17

sulphate per kg. There was no evidence that the A.C.-equivalent was affected by stimulation of the nerve in any of these experiments.

Henderson and Roepke [1933] have made similar experiments, and reached similar conclusions. These results are recorded here because Beznak [1932] has obtained very different results. His figures for the total A.C.-equivalent of normal glands mostly lie between 0·2 and 0·4γ, and are thus much lower than ours. In some of his experiments extracts of active glands had an action on the frog's heart corresponding to ten times as much acetylcholine as the extracts of resting glands, but his highest figure for an active gland is still lower than our lowest figure for a resting gland. The reasons for these discrepancies are still uncertain, but we believe that they are connected with the fact that the frog's heart is affected by a large number of substances in tissue extracts besides acetylcholine, and that some of these substances are antagonistic to acetylcholine.

We have also studied the effect, on the A.C.-equivalent of extracts of salivary glands, of the degenerative section of parasympathetic nerves. The chorda was divided and a portion of the chorda-lingual nerve was removed with aseptic precautions in seven dogs. At intervals varying from 24 hours to 69 days later the dogs were anæsthetized with ether and the submaxillary glands were exposed. The two glands were removed almost simultaneously and immediately placed each in a weighed beaker containing 10 p.c. trichloroacetic acid, cut up with scissors, and weighed. The extract was then prepared in the usual way and tested against acetylcholine on the frog's rectus in the presence of eserine. The results of these experiments are shown in Table V.

TABLE V. Showing the A.C.-equivalent of dogs' submaxillary glands. Normal glands compared with those removed after degenerative section of the chorda.

Days after section of chorda	γ per g. of wet tissue		γ per gland	
	Normal	Denervated	Normal	Denervated
1	1·90	0·90	11	5
6	1·25	1·25	12	9
13	1·50	0·75	11	5
14	3·30	2·40	28	19
17	1·00	1·00	11	6
44	3·30	1·30	22	7
69	2·50	1·60	28	14
Controls:				
Sympathectomy 13 days previously	4·1	4·1	25	26
Both glands normal	2·8	—	13	—
	3·0	—	14	—

In confirmation of earlier work [Langley, 1885; Bradford, 1888] we found that this operation caused a loss of weight of the gland. The mean loss was 18 p.c. $\pm$ 5 (S.D. of mean). In every case the whole denervated gland contained definitely less A.C.-equivalent than the whole normal gland. In every case except two, the concentration was diminished. In the two exceptional cases, although an absolute loss of A.C.-equivalent occurred, the concentration of activity in the gland was apparently unaffected. In a control experiment in which both glands were normal, no significant difference in A.C.-equivalent could be detected. In another experiment the sympathetic nerve supply to the gland was interrupted by excision of the superior cervical ganglion. Extracts of both glands were prepared 13 days later, and again no significant difference in A.C.-equivalent could be detected.

It is obvious, without calculation, that the section of the chorda caused a significant fall in the total A.C.-equivalent of the gland, but it is not obvious whether it had a significant effect on the A.C.-equivalent per g. The percentage fall of A.C.-equivalent per g. in each experiment was therefore calculated. The mean of these values was 33 p.c., and the probability obtained from Student's table of t, that this represented a significant effect was between 0·98 and 0·99 [see Fisher, 1925]. There is thus very little doubt that denervation diminished not only the total A.C.-equivalent of the gland, but also its concentration.

The change involved the loss of about half the activity and did not appear to be progressive after the first 24 hours. Many of the nerve cells in the course of the secretory fibres of the chorda tympani lie in the submaxillary ganglion at the hilum of the gland, but others are scattered along the course of the nerve [Langley, 1898], so that it is probable that in our experiments some preganglionic and some postganglionic fibres were divided. In order to discover whether section of these two classes of nerve fibre would produce different effects, we have carried out some experiments on the parotid glands. The nerve cells in the course of the secretory fibres to the parotid gland are believed to lie in the otic ganglion, and these fibres can therefore be divided peripherally to the ganglion. These experiments were carried out simultaneously with two of the experiments recorded in Table V. The branch of the auriculo-temporal nerve which joins the branch of the facial nerve on the lateral surface of the masseter, was traced centrally to beyond the point where the nerve to the parotid gland leaves it, and excised: 13 and 17 days later the parotid glands were dissected out and extracted in the same way as the submaxillary glands. It was not found possible to remove these glands so

completely as to obtain a reliable estimate of their total A.C.-equivalent, and we contented ourselves with a comparison of the concentrations. In the first dog the concentrations of A.C.-equivalent in the normal and denervated glands were 0·6 and 0·24γ per g., and in the second dog the corresponding figures were 0·3 and 0·12γ. In each case, therefore, there was a loss of about 60 p.c. of the A.C.-equivalent per g. of gland. This loss is not significantly different from that observed in submaxillary glands, so that these experiments have failed to prove that there is any difference in the reaction of salivary glands to the degenerative section of preganglionic and postganglionic nerve fibres.

Again our results stand in contrast to those of Beznak [1932] who observed no change in the A.C.-equivalent of the gland as a result of degenerative section of the nerves.

Discussion.

Acetylcholine cannot be present as such, freely diffusible in tissues, in the quantities suggested by the pharmacological determination of the A.C.-equivalent of extracts. In the first place the concentration present would be more than sufficient to produce large effects on many of the tissues concerned. In the second place, the acetylcholine would be rapidly destroyed by choline esterase unless it were protected from this enzyme in some way.

In explanation of these facts, it has been suggested that the substance whose concentration is measured in experiments of this kind is not a choline ester at all, but some inactive precursor which is not attacked by the enzyme. An alternative theory is that the choline ester is present as such, but is stored in some special structure which prevents it from diffusing freely through the tissue. The fact that mincing the tissue leads to the disappearance of A.C.-equivalent favours this second theory, but whether this is true or not, the question of whether the methods of extraction used are liable to cause the formation of choline esters from some inactive precursor cannot be decided at present. We do not yet know how choline esters are formed in tissues.

The question as to whether the stimulation of the nerves causes the new formation of A.C.-substance, or merely liberates it, has already been mentioned. In the former case it might be expected that, after stimulation, the A.C.-equivalent would increase; in the latter case it should be diminished. There is some evidence, discussed above, of an increase of the A.C.-equivalent of the heart as the result of stimulating the vagus.

In the case of the submaxillary glands, there is as yet no conclusive evidence either way. Gibbs and Szelöczey [1932*b*] found that stimulation for 30 sec. of the nerve to a dog's submaxillary gland, perfused with salt solution, caused the appearance of 0·02–0·05γ of A.C.-equivalent in the perfusion fluid. On re-injection this was sufficient to cause the secretion of about half the quantity of saliva originally secreted as a result of stimulating the nerve, and it is therefore probable that the total amount originally liberated in half a minute was not more than 0·1γ. The results given above show that the total amount normally extractable from the gland is 10–30γ, and this would clearly be sufficient to keep the perfused gland active for a considerable time. If these results are applicable when the blood supply to the glands is normal, it is clear that no very marked change in the A.C.-equivalent in either direction can be expected as the result of stimulating the nerve for periods of less than half an hour. In the experiments described above stimulation was continued for this period, and longer, without effect. This is not really surprising, since it is quite possible that the A.C.-substance was replaced as rapidly as it was liberated.

Similar arguments may be applied to the heart. In an experiment of Witanowski's [1925] an extract corresponding to only 1·3 p.c. of a single heart produced a very striking effect when applied to another heart. The fact that, even when the nerve has not been stimulated, these tissues contain such large quantities of extractable A.C.-substance tempts us to put forward the teleological argument that it is easier to suppose that the direct effect of stimulation of the nerves is the liberation of the active substance already present, rather than the addition of fresh activity to an already ample store.

In the experiments shown in Table V, the A.C.-equivalent of the whole gland was approximately halved as the result of degenerative section of the chorda. This might be due to the degeneration of the hypothetical structure which contains the A.C.-substance, but if this is so, the effect is surprisingly rapid in appearance and surprisingly incomplete. It is also possible that the A.C.-equivalent is normally kept up by nerve impulses passing down the chorda, and that when these impulses are cut off, the production of A.C.-substance still continues, but at a slower rate. In any case these results show that the paralytic secretion which occurs after section of the chorda is not due to an excess of A.C.-substance in the gland.

Summary.

1. Pharmacological methods for detecting choline esters in tissue extracts are discussed. The most satisfactory test objects are the frog's rectus abdominis and the longitudinal muscle of the leech.

2. Many choline esters can only be distinguished pharmacologically from one another by carrying out several assays by different methods and comparing the results quantitatively with one another.

3. An unidentified substance (*R*) occurs in some extracts, which produces an effect on the isolated frog's heart superficially similar to that of acetylcholine, but not affected by atropine.

4. The apparent acetylcholine chloride contents (A.C.-equivalents) of a number of tissues have been measured. The finding by Kapfhammer and his co-workers of large quantities of acetylcholine in blood and other tissues has been confirmed only as regards the human placenta. In other cases the amounts detected have been comparatively small. In this respect our conclusions agree with those of most other workers.

5. Degenerative section of the parasympathetic nerves to salivary glands causes the disappearance of about half of their A.C.-equivalent in 24 hours.

6. Methods of confirming the pharmacological identification of acetylcholine are discussed. There is no reason to doubt that most of the extracts which we prepared did actually contain acetylcholine.

It is a pleasure to acknowledge the help which Dr H. W. Dudley has given us in this investigation.

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INSULIN AND ADRENALINE.

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The suggestion has been put forward recently that certain effects following the administration of insulin to the intact animal might be due to the secondary secretion of adrenaline (Cori [1931], Corkill [1930]). Whilst the evidence appears good that the secretion of adrenaline is stimulated when the blood sugar falls to hypoglycæmic levels, such a view would suggest almost as a corollary that certain effects of adrenaline might be due to insulin and that, as far as the intact animal is concerned, an impasse would be reached in elucidating the essential actions of these hormones. These matters become particularly involved when it is found that certain results of the injections of both adrenaline and insulin appear to be identical, at least qualitatively. Thus, both hormones can, under properly controlled conditions, produce an accumulation of glycogen in the liver of the starving young rabbit (Corkill [1930], Sahyun and Luck [1929], Bischoff and Long [1930], Goldblatt [1929]). This effect was found by some workers to be accompanied by a loss of muscle glycogen in both cases.

General agreement has been reached that insulin stimulates the peripheral oxidation of carbohydrate, and there appears to be good evidence that adrenaline depresses it. This latter view is based on the fact that considerable and long-lasting hyperglycæmia can be produced in the presence of small quantities of hepatic glycogen, such hyperglycæmia in the uninjected animal requiring the infusion intravenously of amounts of glucose far in excess of that available in the liver. It is therefore concluded that two factors make persistent adrenaline-hyperglycæmia possible, viz. inhibition of peripheral oxidation and resynthesis of the products of the breakdown of peripheral glycogen when they

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reach the liver, so that, provided no loss occurs by way of the kidneys, there is a cycle of changes which sustains the blood sugar above normal. The claim that certain effects of insulin are due to adrenaline is particularly directed to those effects which do not accord well with the generally accepted view of the action of insulin. There is no doubt that, in the intact animal, the effects obtained at any moment from any treatment with a hormone is necessarily complicated by the simultaneous action of other internal secretions. Even the injection of an excess of one hormone does not remove the difficulty of quantitative interpretations. The conception of homoiostasis implies that the internal adjustments in the organism make the intact animal unsuitable for the investigation of the essential action of hormones. Qualitatively, however, much can be obtained by the use of the intact animal. For example, no complication of the action of adrenaline by the secondary secretion of insulin could explain adrenaline hyperglycæmia, nor could the rise in oxygen utilization and R.Q. following the injection of insulin be regarded as due to the simultaneous action of adrenaline. In the majority of animals the effect of insulin is to deplete the hepatic glycogen, even when the animal is actively absorbing sugar. Now insulin without doubt brings about a deposition of glycogen in the liver of the depancreatized or phlorizinized animal, but it would not be suggested that this is due to a secondary secretion of adrenaline. Indeed such secondary secretion might be invoked to explain those cases in which insulin depletes liver glycogen, this hormone being of importance in making the glycogen available for the increased oxidation produced by the insulin. A view which has not received attention is that increased peripheral oxidation might reasonably stimulate the endogenous formation of glycogen in the liver.

The questions which will be considered in this paper are:

(1) Is the redistribution of glycogen in young rabbits after the injection of insulin similar to that after the injection of adrenaline?

(2) Is the loss of muscle glycogen after insulin sufficient to account for the increase in liver glycogen which occurs in the same conditions?

(3) Is the action of insulin in these animals associated with a definite increase in blood lactate, the latter being regarded as evidence of the secondary secretion of adrenaline?

(4) Can the effect of insulin be dissociated from that of adrenaline by means of ergotamine or iodoacetic acid?

Experimental.

Glycogen was estimated by a modification of Pflüger's method similar to that described by Lovatt Evans and his co-workers [1931]. The lactate in blood was estimated by the method of Friedmann, Cotonio and Shaffer and blood sugar by Maclean's method.

The animals used in these experiments were mainly young rabbits. The results were compared for litter mates only and, unless otherwise stated, the values for muscle glycogen represent those for mixed samples taken from all four limbs. Where values are given for the total glycogen in the animal, liver and muscles only are meant, the assumption being that the muscles constitute about 50 p.c. of the body weight. All estimations of tissue glycogen are subject to some doubt if referred back to the living animal. The recent findings of Anderson and Macleod [1930] indicate, however, that the rate of loss of glycogen from the muscles post-mortem is very slow if the muscles are uninjured. To test this point in young rabbits they were killed by a sharp blow at the base of the skull, extended and the lumbar spine transected immediately. The gastrocnemius, tibialis anticus and vastus internus were carefully dissected out from the right limb and worked up for glycogen; the animal was left at room temperature (unless otherwise stated in the table) and the corresponding muscles on the left side removed after the times stated in Table I. As Anderson and Macleod found in cats, there is a definite discrepancy between the glycogen of the muscles both on the same side and on opposite sides, but the averages are very close. As is seen in Rabbit No. 6, injury to the muscles leads to marked loss in glycogen. Rather a large loss occurred in No. 7 when the limb was kept immersed in saline at 37° C. It is also seen that the effect of adrenaline on muscle glycogen ceases immediately after death. It is clear that reliance may be placed on glycogen values for muscle even up to an hour after death, provided that (1) averages are taken, (2) the muscles are not injured, and (3) the temperature be kept well below body temperature.

As to the loss of liver glycogen after removal of the liver from the body, Lovatt Evans *et al.* [1931] have shown that, when kept at room temperature, the sliced liver loses 5 p.c. of its glycogen in 1 min. and 40 p.c. in about 8 min., after which the rate of loss is very slow. In similar experiments we have found a similar rate. In the experiments in this paper the liver samples were in the hot potash in about a minute after removal from the body, so that it was not considered necessary to correct.

TABLE I. Post-mortem changes in glycogen content of mammalian muscle.

Rabbit No.	Wt. kg.	Right p.c.	Left p.c.	Remarks and time between muscle removal
1	1·26	0·177	0·165	1 hour
		0·206	0·211	
		0·190	0·231	
		Av. 0·191	Av. 0·202	
2	1·50	0·211	0·332	1 hour
		0·251	0·208	
		0·185	0·159	
		0·219	0·233	
3	1·35	0·139	0·093	0·25 mg. adrenaline intravenously
		0·114	0·065	2 min. before death 1 hour
		0·151	0·126	
		0·135	0·095	
4	1·65	0·202	0·140	As in No. 3
		0·189	0·151	
		0·117	0·175	
		0·169	0·155	
5	1·65	0·368	0·408	0·5 mg. adrenaline intravenously
		0·396	0·461	2 min. before death ½ hour
		0·348	0·457	
		0·371	0·442	
6	1·00	0·639	0·450	Muscles on left side were bruised
		0·534	0·394	1 hour
		0·397	0·451	
		0·523	0·432	
7	1·20	0·323	0·250	Left limb kept at 37° C. after
		0·262	0·154	death 1 hour
		0·234	0·199	
		0·276	0·201	

The effect of adrenaline on glycogen distribution in young rabbits.

The suitability of these animals for comparative work on glycogen distribution has been demonstrated by the present writer [1929]. It is important that such comparisons be made between litter mates, preferably bred in captivity. All the animals we have used have been so bred. A specially constructed light wooden collar was used during starvation of the animals, to make ingestion of fæces impossible. No difficulty has been found in obtaining animals with empty stomachs in this way if the diet during a day or two before the experimental period was somewhat restricted. In Table II are given the results of subcutaneous injection of adrenaline into these animals after starvation for various periods.

It is seen that in the starving young rabbit there is a considerable uniformity in the total glycogen per unit mass, even after greatly varying

TABLE II. Effect of adrenaline on glycogen distribution in young rabbits.

Litter No.	Time of starvation (days)	Animal No.	Wt. g.	Blood sugar mg./100 c.c.	Glycogen: Liver p.c.	Glycogen: Muscle p.c.	Glycogen: Total g./100 g. body wt.	Dose and time of action of adrenaline
1	2	1	587	175	0·55	0·41	0·22	Control
		2	675	120	0·50	0·36	0·20	Control
		3	653	360	0·75	0·24	0·14	0·2 mg. 3 hours
		4	640	390	0·26	0·16	0·09	0·3 „ 3½ „
		5	660	380	0·32	0·35	0·18	0·4 „ 3½ „
		6	677	360	0·37	0·21	0·11	0·5 „ 4 „
2	2	1	785	156	0·31	0·34	0·18	Control
		2	767	102	0·27	0·27	0·14	Control
		3	615	340	0·28	0·26	0·14	0·5 mg. 3½ hours
		4	775	300	0·60	0·18	0·11	1·0 „ 3½ „
		5	710	364	0·43	0·15	0·18	1·0 „ 3½ „
		6	853	330	0·16	0·15	0·08	2·0 „ 3½ „
3	2	1	495	200	0·52	0·26	0·15	Control
		2	432	109	0·50	0·26	0·14	Control
		3	437	41	1·60	0·38	0·25	Insulin 12 units in 6 doses over 6 hours
		4	485	171	0·29	0·18	0·11	1·0 mg. repeated after 2 hr. 25 min. 6 hours
4	3	1	445	77	0·41	0·36	0·19	Control
		2	408	—	0·15	—	—	0·2 mg. 1½ hours
		3	457	350	0·26	0·19	0·10	1·1 mg. in 4 doses 4½ hours
5	5	1	550	95	1·30	0·33	0·20	Control
		2	518	264	0·24	0·28	0·15	1·1 mg. in 4 doses 4½ hours
		3	490	350	0·35	0·27	0·15	As in 2

periods of starvation. The average of the controls was $0{\cdot}18 \pm 0{\cdot}04$ g. per 100 g. animal, whilst the average for the animals injected with adrenaline was $0{\cdot}13 \pm 0{\cdot}05$. We have introduced in Litter 3 an animal injected with large doses of insulin in order to illustrate the vastly different effect produced in these animals by this hormone. The average weight of these rabbits is about 500 g. and the average loss of glycogen per 100 g. is about 0·05 g., *i.e.* an average absolute loss of about 0·25 g. per animal. There is no reason to believe that this is brought about by increased oxidation or by loss in the urine (very little urine is as a rule excreted in these experiments). We are led, therefore, to the probability that there is a storage of the degradation products of glycogen actually in the tissues after the injection of adrenaline. Whilst the acute effect of adrenaline is a loss of both liver and muscle glycogen, the process of recovery is a peculiar one. It has been known from the time of Claude Bernard that muscle glycogen does not produce glucose but lactic acid.

Hence, loss of muscle glycogen is due either to increased oxidation or to increased formation of lactic acid. That adrenaline produces an increase in circulating lactate has been shown by several workers, notably by Cori. Since it is known that lactic acid can give rise to hepatic glycogen, the following cycle of changes is taken as occurring after the injection of adrenaline:

Muscle glycogen → Lactic acid → Hepatic glycogen → Blood glucose → Glycosuria if threshold is exceeded.

Using rats which (*a*) had very small initial liver glycogen values, and (*b*) were injected with small doses of adrenaline, Cori found that the liver glycogen was very considerably increased and the muscle glycogen fell. Pollak [1909] had already observed that such an end-effect could occur after adrenaline injected in large and repeated doses during starvation.

The following is an experiment on the same lines as Pollak's: Two rabbits from the same litter were starved for 3 days and then for a further 2 days, during which one of them received injections of adrenaline and the other was kept as control. The injected animal received 2 mg. adrenaline a day and at the time of death had excreted 1·5 g. glucose. On analysis the control gave a blood sugar of 0·056 p.c. and a liver glycogen 0·30 p.c.: the injected animal gave a blood sugar of 0·40 p.c. and a liver glycogen of 2·5 p.c.

More recently Corkill [1930], using small doses of adrenaline, demonstrated the same effect in young rabbits. Sahyun and Luck [1929]

TABLE III.

Rabbit No.	Wt. g.	Blood: Sugar mg./100 c.c.	Blood: Lactate mg./100 c.c.	Glycogen: Liver p.c.	Glycogen: Muscle p.c.	Glycogen: Total g./100 g. body wt.	Time after adrenaline hours
			Controls				
1	775	117	76	0·490	0·158	0·096	—
2	800	113	87	0·859	0·126	0·092	—
3	950	102	71	0·489	0·187	0·106	—
						Av. 0·097	
			Adrenaline 0·15 mg.				
4	875	150	63	1·058	0·101	0·108	2·0
5	800	216	93	1·524	0·101	0·103	2·2
6	950	164	74	1·352	0·144	0·120	2·4
						Av. 0·110	
			Adrenaline 0·15 mg. and pituitrin 1 c.c.				
7	750	213	89	1·457	0·079	0·092	2·3
8	900	177	79	1·920	0·083	0·112	2·5
9	800	187	111	1·528	0·094	0·103	2·7
						Av. 0·102	

found that 1 mg. of adrenaline produced a rapid fall in the glycogen of the liver and the muscles, the former recovering and attaining very high values, whilst the latter remained at extremely low levels for as long as 42 hours. This must mean that the lactic acid produced from the muscle lies dormant until such time as the liver is able to work it up to glycogen. If, however, small doses of adrenaline are used it is possible to obtain an almost quantitative redistribution of glycogen from the muscles to the liver in between 2 and 3 hours. This is shown in Table III.

The effect of a non-glycosuric dose of adrenaline.

A litter of nine young rabbits was starved for 24 hours. Three were taken as controls, three received 0·15 mg. adrenaline subcutaneously and the remaining three received 1 c.c. pituitrin and 25 min. later 0·15 mg. adrenaline. The last three animals were so injected to see if, as has been stated by some workers, pituitrin exerts an antagonistic effect on adrenaline hyperglycæmia: such an effect was not found.

It is clearly seen that, with this dose of adrenaline, there was no loss in glycogen, a quantitative redistribution having occurred (Table III).

Time factor in the resynthesis of liver glycogen.

The time factor in the glycogen redistribution is shown in Table IV, where it is seen that the fall in muscle glycogen after 0·5 mg. adrenaline had reached a maximum in about 1½ hours and from that time on the

TABLE IV. Litter of five young rabbits. 48 hours' starvation before injection.

Rabbit No.	Wt. g.	Blood sugar mg./100 c.c.	Glycogen Liver p.c.	Glycogen Muscle p.c.	Glycogen Total g./100 g. body wt.	Dose of adrenaline and time of action
1	942	380	0·51	0·28	0·16	0·5 mg. 1½ hours
2	985	139	2·87	0·23	0·20	,, 4½ ,,
3	865	125	2·59	0·29	0·24	,, 12 ,,
4	980	129	1·14	0·28	0·18	,, 18 ,,
5	655	152	2·53	0·35	0·26	,, 24 ,,

products of the breakdown of glycogen were being resynthesized in the liver, the rate of glycogenolysis in the liver having become normal in about 4½ hours.

Summary of experiments on the effect of adrenaline.

We find that the end-effect of adrenaline depends on the dose and the time of action. With a large enough dose the effect in young rabbits even up to 3 hours after the injection may be loss of glycogen from both

the liver and muscles, but after this time the liver glycogen recovers and far exceeds the initial value although the muscle glycogen may fall no further. This is clearly due to a lag between the liberation of the products of the breakdown of muscle glycogen and their resynthesis in the liver. With a suitable smaller dose of adrenaline the glycogenolytic effect in the liver may be so slight that resynthesis proceeds so rapidly that quantitative redistribution is attained in a relatively short time. This would involve the resynthesis of the glucose liberated from the liver when it reaches the muscles, an effect which is usually associated with the action of insulin.

We proceed now to present results illustrating the effects of insulin in these animals and to examine how far it is justifiable to consider that adrenaline, secondarily secreted, is responsible for them.

The action of insulin in young rabbits.

The injection of insulin is followed by an increase in the peripheral oxidation of carbohydrate and in the majority of normal animals this leads to a loss in both muscle and liver glycogen. Corkill [1930] found this to be the case in several different species. To his list of animals we may add the guinea-pig with which the results in Table V were obtained. Ten small guinea-pigs were starved for 24 hours. The muscles used for glycogen estimation were the tibialis anticus and the gastrocnemius; usually about 2 g. could be obtained.

The results shown in Table V are puzzling. The effectiveness of the insulin is clear from the blood-sugar values. No significant change in the

TABLE V. Young guinea-pigs starved 24 hours.

Rabbit No.	Wt. g.	Blood sugar mg./100 c.c.	Glycogen Liver p.c.	Glycogen Muscle p.c.	Dose of insulin and time of action
1	214	135	0·440	0·619	Controls
2	258	144	0·366	0·619	
3	600	97	0·312	0·552	
4	550	85	0·292	0·631	
5	350	58	0·509	0·310	
6	183	47	0·457	0·412	1 unit 3 hours
7	287	27	1·074	0·377	1½ „ 3½ „
8	550	35	0·365	0·770	1½ „ 2½ „
9	550	60	0·396	0·559	1 „ 3½ „
10	400	0	0·412	0·681	2 „ 2½ „
		Averages: Controls ...		0·384	0·546
		Treated animals		0·408	0·559

(Anomalous liver value for No. 7 omitted in average.)

liver or muscle glycogen was found in spite of a fall in average blood sugar from 104 to 34. Further study of this species will be necessary.

It is, however, with the young rabbit that the striking and apparently paradoxical action of insulin is observed, viz. a very marked increase in liver glycogen, which exceeds any loss which may simultaneously occur in the muscles. In Table VI are shown the results in a typical experiment. Calculated in the usual way, the average total glycogen per 100 g. body

TABLE VI. A litter of eight young rabbits starved for 24 hours. The dose of insulin was 1 unit injected subcutaneously.

		Blood		Glycogen			
Rabbit No.	Wt. g.	Sugar mg./100 c.c.	Lactate mg./100 c.c.	Liver p.c.	Muscle p.c.	Total g./100 g. body wt.	Time of action
1	800	168	44	0·549	0·202	0·118	Controls
2	625	156	37	0·552	0·198	0·116	
3	700	143	53	0·613	0·181	0·110	
							hr. min.
4	825	88	63	1·804	0·130	0·130	1 50
5	750	106	50	2·647	0·139	0·150	2 10
6	800	84	68	5·042	0·141	0·245	2 55
7	775	96	44	2·824	0·129	0·162	3 15
8	725	106	68	3·426	0·098	0·166	3 30

weight for the controls in Table VI is 0·115 g. and for the injected animals 0·171 g. Certain workers, confirming the increase in liver glycogen here demonstrated, attribute it to the secondary secretion of adrenaline. It will be seen in Table VI that the blood sugar of the injected animals was at an average normal level, this being due to the fact that this litter was of the kind, sometimes met with, in which the blood sugar is high. Can we on the basis of the blood sugar consider that adrenaline was being secreted at a rate sufficient to account for the liver glycogen? In the dog the secondary secretion of adrenaline occurs according to Houssay, Lewis and Molinelli [1924] at a blood-sugar level of 0·05 p.c., and according to La Barre and Houssa [1932] at 0·075 p.c. The rabbit does not lend itself to determinations of this sort, but it is improbable that the critical level is as high as that of the injected animals in this experiment. On the other hand, the average blood lactate rose from 45 to 59, which might be regarded as slight evidence of an increased secretion of adrenaline; but Matakas [1932], in a recent paper, reports that the rabbit is not reliable in this connection. Averages from young litter mates are, in our experience, reasonably reliable.

The decisive experiment would be to observe the effect of insulin in the adrenalectomized young rabbit. We have, after many attempts,

failed to obtain clear results by this method, since the animals do not survive the operation for a sufficiently long time.

Two other ways of attacking the problem seemed possible, viz. by using ergotamine and iodoacetate. The former is known to inhibit adrenaline hyperglycæmia, and might conceivably act similarly on the peripheral liberation of lactate. If it did so, then it might be possible to dissociate the characteristic effects of adrenaline from those of insulin. Iodoacetate is known to prevent the post-mortem liberation of lactic acid in muscle and might, in adequate dose, prevent the liberation of lactic acid produced by adrenaline in the living animal.

Ergotamine and the action of insulin.

The ergotamine was used in the form of "ergotamine methan sulphonate" [Sandoz] injected intravenously and the insulin was injected as usual subcutaneously. The animals were from one litter and were starved for 24 hours. The results are given in Table VII, and it will

TABLE VII. A litter of four young rabbits, starved for 24 hours.

				Blood		Glycogen			
Rabbit No.	Wt. g.	Insulin unit	Ergotamine mg.	Sugar mg./100 c.c.	Lactate mg./100 c.c.	Liver p.c.	Muscle p.c.	Total g./100 g. body wt.	Time of action
1	784	0	0	134	33	0·267	0·148	0·081	—
									hr. min.
2	715	1·0	0	65	36	0·933	0·118	0·091	2 45
3	638	0·8	4·0	23	33	1·293	0·158	0·130	2 7
4	592	0·8	2·5	14	30	0·967	0·237	0·153	2 2

suffice to say that the effect shown in Table VI is not inhibited by ergotamine. The effectiveness of the ergotamine is shown by the much greater and more rapid fall in blood sugar in the animals injected with it. It will also be observed that the blood lactate remained the same in all the animals.

Ergotamine and the action of adrenaline.

The results of the combined actions of ergotamine and adrenaline are shown in Table VIII. It is sufficient to state that ergotamine, in doses large enough to inhibit adrenaline hyperglycæmia, does not prevent the redistribution of glycogen which has been demonstrated in Table III. It will also be observed that, whilst as a result of the ergotamine the adrenaline only raised the average blood sugar by about 16 p.c., the rise in lactic acid in the blood was over 80 p.c.

TABLE VIII. A litter of ten young rabbits, starved for 24 hours.

Rabbit No.	Wt. g.	Ergotamine mg.	Adrenaline mg.	Blood mg./100 c.c. Sugar	Blood mg./100 c.c. Lactate	Glycogen Liver p.c.	Glycogen Muscle p.c.	Glycogen Total g./100 g. body wt.	Time of action of adrenaline
1	625	0	0	135	23	0·754	0·430	0·204	—
2	625	0	0	115	17	0·567	0·202	0·119	—
3	525	0	0	111	16	1·164	0·193	0·130	—
4	700	0	0	137	39	1·275	0·204	0·204	—
			Averages	125	24	0·940	0·292	0·164	
									hr. min.
5	650	1·3	0·15	171	40	1·751	0·154	0·140	2 15
6	625	1·25	0·15	129	43	3·126	0·231	0·219	2 35
7	750	1·5	0·15	137	40	2·354	0·125	0·138	2 55
8	650	3·3	0·15	139	28	1·043	0·130	0·098	2 30
9	900	5·0	0·30	152	56	2·192	0·120	0·126	2 50
10	750	4·2	0·20	146	56	2·041	0·188	0·158	3 15
			Averages	146	44	2·085	0·158	0·147	

Nos. 5, 6, 7 received the adrenaline 10 min. after the ergotamine, Nos. 8, 9, 10, 1 hour after it.

Summary of experiments on the influence of ergotamine.

Ergotamine does not prevent the increase in liver glycogen produced by insulin in the starving young rabbit, this increase being greater than can be accounted for by losses of glycogen from the muscles. Ergotamine, in doses sufficient to inhibit adrenaline hyperglycæmia, does not prevent the liberation of lactic acid from the muscles and the quantitative redistribution of glycogen which follows the injection of a small dose of adrenaline. It appears, therefore, that the mechanism of glycogenolysis in the liver is more rapidly paralysed by ergotamine than is that in the muscles. If, however, the dose of ergotamine is sufficient demonstrably to paralyse the peripheral vaso-constrictors in the anæsthetized animal, then the increase of lactate in the blood caused by adrenaline is also prevented (Goldblatt [1933]). Thus, with the doses of ergotamine we have used, we have been unable to dissociate the effects of insulin and adrenaline.

Iodoacetate and the actions of insulin and adrenaline.

The dose of the neutralized acid which could safely be given was first determined. The acid was neutralized with $N/2$ NaOH, using methyl orange as indicator.

(*a*) Rabbit. 1·1 kg.
5 mg. intravenously ... ... No symptoms
15 mg. 1 hour later ... ... No symptoms
40 mg. 1¼ hours later ... ... Convulsions and death in 15 min.
(*b*) Rabbit. 1·0 kg.
10 mg. intravenously ... ... No symptoms
15 mg. 55 min. later ... ... No symptoms

It was decided to use doses up to 15 mg.

The results are set out in Tables IX and X and may be summarized as follows:

(1) Iodoacetate in doses up to 10 mg. does not seriously interfere with the accumulation of liver glycogen or the increase in total body glycogen which we have seen constantly to occur in these animals after insulin.

(2) With 15 mg. this effect is inhibited to a very great extent but not abolished, the total glycogen per unit body weight remaining unchanged. This amount of iodoacetate does not interfere with the rate of fall in blood sugar, nor is there any appreciable change in blood lactate.

(3) 15 mg. iodoacetate prevent the rise in liver glycogen which normally follows the injection of small doses of adrenaline but do not prevent the hyperglycæmia, the rise in lactic acid in the blood or the fall of muscle glycogen. The inability of the liver to synthesize the liberated lactic acid is reflected in the persistence of a high blood lactate. The total glycogen per unit body weight is very greatly diminished (cf. Table III).

TABLE IX. Fifteen young rabbits from two litters of approximately the same age were starved for 24 hours. Of these six were used as controls, two were injected with insulin alone, and seven with both insulin and acetoacetate as detailed in the table. Except for one animal in the last group, the litters were equally represented in all the groups.

Rabbit No.	Wt. g.	Iodo-acetate mg.	In-sulin unit	Blood mg./100 c.c.		Glycogen			Time of action of insulin hours
				Sugar	Lactate	Liver p.c.	Muscle p.c.	Total g./100 g. body wt.	
1	700	0	0	77	40	0·304	0·181	0·100	—
2	700	0	0	95	39	1·057	0·189	0·130	—
3	550	0	0	100	84	0·397	0·152	0·087	—
4	700	0	0	90	68	1·949	0·267	0·199	—
5	500	0	0	67	62	0·514	0·197	0·117	—
6	600	0	0	72	63	1·096	0·226	0·150	—
			Averages	83	59	0·886	0·202	0·131	
7	750	0	1	30	40	2·169	0·283	0·210	2·3
8	925	0	1	54	46	3·947	0·402	0·323	2·3
			Averages	42	43	3·008	0·342	0·267	
9	800	5	1	58	59	2·587	0·250	0·216	2·3
10	850	10	1	30	89	2·156	0·212	0·179	1·3*
			Averages	44	74	2·372	0·231	0·198	
11	650	15	1	30	31	1·526	0·258	0·178	2·3
12	750	15	1	48	68	0·809	0·160	0·108	2·3
13	725	15	1	62	48	1·135	0·136	0·109	2·3
14	700	15	1	48	46	0·875	0·173	0·116	2·3
15	700	15	1	45	74	1·469	0·138	0·130	2·3
			Averages	48	53	1·163	0·173	0·128	

* Convulsion.

TABLE X. Eight young rabbits from the same litter were starved for 24 hours. Three were used as controls and five were injected with iodoacetate and adrenaline.

Rabbit No.	Wt. g.	Iodo-acetate mg.	Adrena-line mg.	Blood mg./100 c.c. Sugar	Blood mg./100 c.c. Lactate	Glycogen Liver p.c.	Glycogen Muscle p.c.	Glycogen Total g./100 g. body wt.	Time of action of adrena-line hours
1	650	0	0	137	56	Lost	0·400	—	—
2	650	0	0	118	28	2·139	0·574	0·358	—
3	650	0	0	113	26	1·065	0·428	0·255	—
			Averages	123	37	1·602	0·467	0·307	
4	600	15	0·15	229	51	1·098	0·194	0·136	2·3
5	600	15	0·15	236	39	1·055	0·245	0·161	2·3
6	750	15	0·15	168	64	2·457	0·239	0·226	2·3
7	600	15	0·15	187	46	1·205	0·258	0·181	2·3
8	625	15	0·15	227	85	1·164	0·232	0·162	2·3
			Averages	209	57	1·396	0·234	0·173	

It is clear that the increase of lactate in the blood caused by adrenaline is not affected by iodoacetate in doses of 15 mg. Hence, were the accumulation of liver glycogen shown in Rabbits Nos. 7 and 8 in Table IX due to resynthesis from lactate released from the muscles, we should have obtained a great rise in blood lactate in Rabbits Nos. 11 to 15, in which the formation of new glycogen in the liver was practically abolished. (Rabbit No. 10, Table IX, illustrated that the peripheral release of lactate was easily possible.) We consider that this failure of the lactate to be increased constitutes evidence against the adrenaline hypothesis. In Table IX the last group of animals, in spite of the fall in blood sugar, showed no change in total body glycogen, whereas in the litter of Table X the total fell by over 40 p.c., no appreciable loss having occurred by the urine. Iodoacetate, whilst not giving the unequivocal evidence sought for, served to emphasize the quantitative differences between the effects of insulin and adrenaline in these animals.

DISCUSSION.

The most recent contribution to the problem presented by these young animals is that of Corkill [1932], in which it is shown that diphtheria toxin, in doses that were too small to abolish the power of the liver to make glycogen from glucose, completely prevented the deposition of liver glycogen which ordinarily follows the injection of insulin or adrenaline. The toxæmia is accompanied by a distinct resistance to insulin as far as the blood sugar is concerned, but this resistance can be overcome by ergotoxine, which supports the view that adrenaline secretion is

increased during the toxæmia. But ergotoxine does not allow of a deposition of liver glycogen by the action of insulin in the toxæmic young rabbit. Corkill has thus also reached an impasse in the elucidation of the source of the new glycogen. In regard to these very interesting experiments it would be important to determine whether in toxæmia there occurred a large rise in blood lactate, and whether injected lactate gave rise to liver glycogen. By intravenous injection of *d*- or *i*-lactate it is easy to produce a deposition of liver glycogen in normal young rabbits, and it may be that the toxæmic liver may have lost the power to form glycogen from lactate whilst retaining that from glucose. Should this prove to be the case, the necessary link in connecting the anomalous action of insulin with the adrenals might become clearer. The fact that, as we have seen, ergotamine permits both the insulin and adrenaline effects to take place and that iodoacetate and diphtheria toxin [Corkill] prevent them, undoubtedly gives qualitative support to the hypothesis that there is some link between the two; but on quantitative grounds we consider that there are outstanding differences between the observed changes and what might reasonably be expected if the effects following the injection of insulin in these animals were really due to adrenaline. Further facts must be obtained before a definite decision can be reached.

Conclusions.

1. Insulin and adrenaline under properly controlled conditions produce a very marked increase in liver glycogen in the starving young rabbit.

2. In the case of insulin this increase may or may not be associated with a fall in muscle glycogen. The effect on the total glycogen of the body is most frequently an increase.

3. In the case of adrenaline in non-glycosuric doses, the increase in liver glycogen is always associated with a fall in muscle glycogen. With a properly adjusted dose there is a quantitative redistribution of glycogen between the muscles and the liver, the total remaining unchanged. With larger doses there is a loss in both situations.

4. Ergotamine, in doses up to 5 mg. intravenously, does not prevent the action of insulin or of adrenaline on the distribution of glycogen.

5. Iodoacetate, in doses of 15 mg., prevents the accumulation of liver glycogen usually produced by insulin and adrenaline in young rabbits. The final result in the case of adrenaline is a loss of glycogen from both liver and muscles; in the case of insulin the total glycogen was not changed.

6. The action of insulin in these animals is not accompanied by such an increase in blood lactate as would justify the belief that the adrenal secretion is the cause of the increase in liver glycogen which always follows such action.

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THE NORMAL BEHAVIOUR OF THE ISOLATED UTERUS OF THE GUINEA-PIG, AND ITS REACTIONS TO ŒSTRIN AND OXYTOCIN.

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The object of this paper is to amplify the substance of two communications by Marrian and Newton [1932, 1933], on the action of œstrin and oxytocin on the uterine contractions of guinea-pigs. As a rule this animal has been adhered to, but mice were employed for a few experiments. The animals have been used in four conditions, viz. immature virgin, mature non-pregnant, and oöphorectomized, and it is proposed to arrange the findings under these four headings.

The method used consisted of suspending the uterus, or a strip of it, in a bath of oxygenated saline solution, at 37° C., and recording its contractions by means of a light lever and frontal writing point. Except where otherwise stated, the animal was killed by a blow on the head, the blood drained from it, and the uterus removed immediately. The solution and apparatus used were those suggested by Burn and Dale [1922].

The immature virgin uterus.

Normal behaviour. The uterine horns of female guinea-pigs of about 200 g. weight, and reared apart from males, are generally regarded as satisfactory for the assay of the posterior pituitary oxytocic principle. This is partly because, when suspended in the muscle bath, they maintain a constant degree of relaxation, their spontaneous contractions being insignificant and irregular. The amplitude of these contractions may be reduced, up to a point, by applying a load to the lever. Whatever be this load, however, after about 2 hours the spontaneous activity of the uterus increases, and the base line of the tracing rises, *i.e.* a certain amount of tonic contraction takes place (Fig. 1).

Action of oxytocin. This is well known, and requires only a brief description. On adding oxytocin to the bath, a strong contraction of the muscle takes place, and for a few minutes a state of steady tonus is maintained. The level of this is constant for the same submaximal dose of oxytocin; if the latter is increased or decreased, the level is respectively higher or lower. This property of the uterus is the basis for the assay of pituitrin, and is fully discussed by Dale and Laidlaw [1912], and Burn and Dale [1922]. If the dose of oxytocin is left in the bath, the tonus falls

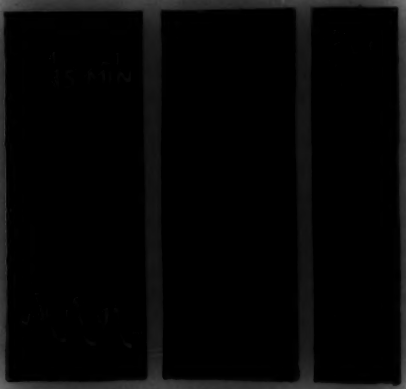

Fig. 1. Virgin guinea-pig, 220 g. Upper and lower tracings taken simultaneously from left and right uterine horns respectively. The solution bathing the right horn was saturated with ketohydroxyœstrin. First space = interval of 1 hour; second space = interval of ½ hour.

after a time, and the tracing is broken by fairly large, irregular, spontaneous contractions. If the solution is changed to a fresh one immediately the contraction has attained its initial steady value, the relaxation is rapid, and the spontaneous contractions no greater than before. After some hours the preparation becomes too sensitive for quantitative work, responding with a maximal contraction to different and formerly submaximal doses of oxytocin.

Action of œstrin. (*a*) Effect on normal behaviour. One horn of the uterus was suspended in the plain saline solution, and the other in a similar solution saturated with pure crystalline ketohydroxyœstrin. There was no significant difference between the spontaneous contractions in the

two cases, and the only difference appeared to be that the increase in tonus and general activity, which normally comes on after a few hours, was delayed in the horn exposed to the œstrin solution (Fig. 1). The

Fig. 2.

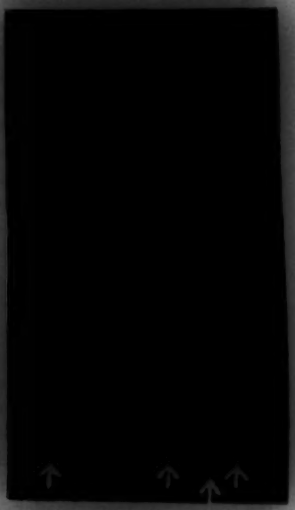

Fig. 3.

Fig. 2. Virgin guinea-pig, 210 g. Contractions produced by addition to bath (60 c.c.) of the following: *A* = 0·016 unit oxytocin + 0·1 c.c. absolute alcohol. *B* = 0·016 unit oxytocin + 0·2 mg. ketohydroxyœstrin in 0·1 c.c. alcohol. *C* = 0·032 unit oxytocin + 0·2 mg. ketohydroxyœstrin in 0·1 c.c. alcohol. *D* = 0·032 unit oxytocin + 0·1 c.c. absolute alcohol. Interval between doses 15 min.; bath washed out after each dose, and drum stopped during relaxation of muscle. The last four contractions show that at least an additional 0·016 unit oxytocin is required to overcome the effect of 0·2 mg. of ketohydroxyœstrin.

Fig. 3. Upper tracing. Virgin guinea-pig 240 g. Contractions: 1, 2, and 5 produced by 0·02 unit oxytocin + 0·1 c.c. absolute alcohol; 3 and 4 by 0·02 unit oxytocin + 0·2 mg. triacetate of trihydroxyœstrin in 0·1 c.c. alcohol.

Lower tracing. Virgin guinea-pig, 200 g. Arrows: 0·1 mg. monomethyl ether of ketohydroxyœstrin in 0·5 c.c. alcohol; 0·04 unit oxytocin; fresh bath, and interval of 30 min.; 0·04 unit oxytocin + 0·05 c.c. absolute alcohol.

reason for this late activity is in any case obscure, and it is doubtful if it is permissible to draw conclusions from the later part of experiments such as these.

(*b*) Effect on reaction to oxytocin. The oxytocin used in these experiments was a partially purified solution prepared by J. M. Gulland ("Kamm solution," Gulland and Newton [1932]). It was diluted, and the amount found which would produce a contraction of suitable size when added to the muscle bath (capacity on this occasion 100 c.c.). It was then ascertained that the simultaneous addition of 0·1 c.c. absolute alcohol was without effect on the response to this dose. Finally a test was performed in exactly the same way as if two different solutions of oxytocin were being compared, except that the dose of oxytocin was constant, and the difference lay in the simultaneous addition of 0·1 c.c. of alcohol in one case, and 0·2 mg. œstrin dissolved in 0·1 c.c. of alcohol in the other. The result of this experiment has previously been reported, namely, that œstrin markedly reduces the response to oxytocin [Marrian and Newton, 1932]. The accompanying tracing (Fig. 2) gives a rough idea of the magnitude of this reduction.

The action of derivatives of œstrin. (*a*) The triacetate of trihydroxy-œstrin. The effect of this is the same as that of ketohydroxyœstrin (Fig. 3).

(*b*) The monomethyl ether of ketohydroxyœstrin. This itself produces a symmetrical contraction and relaxation of the muscle. In investigating its effect on the response to oxytocin, this necessitates some irregularity in the timing of the doses, but so far as these experiments warrant any conclusion, it is that the response to a dose of oxytocin, after the contraction from the methyl ether has subsided, is depressed (Fig. 3).

Effect of pregnandiol. The contractions from oxytocin are slightly diminished in the presence of pregnandiol, but when this has been removed, the original degree of contraction with oxytocin cannot be reproduced. It is therefore impossible to say whether this is a permanent, slight effect of pregnandiol, or whether the sensitivity of the uterus is gradually diminishing, and the pregnandiol is inactive. The same result was obtained, however, on several occasions. In any case, the action of pregnandiol is not comparable with that of œstrin.

Discussion.

These experiments throw little light upon the virgin uterus as an organ; the ultimate effect of oxytocin is simply to increase its muscular activity, but this is as irregular as before. Œstrin has at the most a questionable depressor influence. Regarded as a means for measuring the activity of oxytocin, however, the uterus assumes an apparent importance, for it is clear that this activity is impaired *in vitro* by the presence

of œstrin, and certain of its derivatives. In studying the interaction between the two hormones, it cannot be too strongly emphasized that results may vary according to the technique adopted and the animal used. The sexual development and condition of the animal may also have an influence. The only experiments bearing on the present work are those previously performed by the same technique. Heller and Holtz [1932] and Jeffcoate [1932] stated that œstrin was without effect on the response to oxytocin of the virgin uterus *in vitro*, and although they confirmed their results with crystalline œstrin, it is possible that they had difficulty in getting a sufficient concentration of this substance into solution. Burn and Bourne reported [1928] that œstrin increased the sensitivity of the uterus to oxytocin, but withdrew their experiments [1932] when crystalline œstrin became available.

The fact that it is now clearly demonstrable that œstrin impairs the action of oxytocin, while it is not so clear that it interferes with the normal behaviour of the uterus, suggests immediately that œstrin may have no action on the muscle *in vitro*, but may throw out of action, by physical or chemical means, a part of the oxytocin. To set against this is the fact that the activity of oxytocin can be accurately measured, whereas it is difficult to interpret what is and what is not variation in the extremely irregular normal contractions, so that a slight effect on the latter may be overlooked.

The uterus of the oöphorectomized animal.

Normal behaviour. The uterus undergoes involution and coincidentally the spontaneous activity dies away, so that 3 months after oöphorectomy a preparation is obtained which is quiescent in the muscle bath. In behaviour it resembles the virgin uterus, except that the spontaneous contractions are smaller and more sluggish; if left in the bath for some time, it may show slow variations in tonus, but does not become active like the virgin organ. Any deviation from this condition is very likely to be due to regeneration of ovarian tissue [Parkes, Fielding and Brambell, 1927], and to guard against this about $\frac{1}{4}$ in. of each uterine horn has been regularly removed with the ovaries.

Action of oxytocin. Addition of a suitable amount of oxytocin to the bath causes a slow contraction of the muscle, which becomes steady at a level corresponding to the dose of oxytocin given. On one or two occasions when the latter was left in the bath for a long time, the contraction of the muscle appeared to be sustained indefinitely. The height of the contraction may not be attained until as long as 8 or 10 min., and after

washing out the bath, the relaxation is correspondingly slow. It was thought that such a stable preparation might be better than the virgin uterus for the assay of oxytocin, and, at the suggestion of Dr A. S. Parkes, a considerable number of experiments was made. It was concluded that the uteri were not sufficiently stable until 3 months after oöphorectomy, and that then estimations definitely could be done; some preparations 4 months after oöphorectomy discriminated clearly between doses differing by only 10 p.c. (Fig. 4). As the comparison of two doses

Fig. 4. Guinea-pig 642 g., oöphorectomized 16 weeks previously. Four successive doses oxytocin, at intervals of 22 min., 0·030, 0·027, 0·027, 0·030 unit respectively. Bath changed twice between doses. Contractions 64–72 p.c. maximal. Duration of each contraction 8·5 min.

(*i.e.* four contractions in all) took in some cases from 2–3 hours, it was decided that the preparation was of little practical value except possibly for confirmatory purposes.

Action of œstrin. The same technique was used as for the virgin uterus, and it was found that here also the response to oxytocin was diminished by œstrin; the ketohydroxy-variety appeared to be slightly more active than the trihydroxy-. The result of a very rough test (Fig. 5), however, seemed to indicate that the activity of the latter was about five times as great as that of the former, as determined on a previous occasion (Fig. 2). The fact that ten times the amount of œstrin was used in the first test suggests that the limit of solubility might have been passed, and that the two tests are not really comparable. They give an idea, however, of the order of the "antagonism" between œstrin and oxytocin. Using the present preparation we were able to demonstrate the depressor activity with respect to oxytocin of an aqueous solution (0·02 mg. per c.c.) of trihydroxyœstrin (Fig. 5).

The results of injection of œstrin, killing the animal 18 hours after the last dose, and putting up the horns in saline, were vitiated by the control

experiments. The horns of the injected animals showed a series of powerful contractions, quite different from the typical uteri of oöphorectomized animals, but the actual control animals used also exhibited an unusual though lesser degree of uterine activity. It is possible, therefore, that in this batch of guinea-pigs (6 months after oöphorectomy) some regeneration of ovarian tissue might have taken place.

Fig. 5. Guinea-pig, 524 g.; oöphorectomized 12 weeks previously. Contractions, in order, produced by addition to bath (100 c.c.) of following: (*a*) 1. 0·01 unit oxytocin. 2, 3, 6. 0·01 unit oxytocin + 0·1 c.c. absolute alcohol. 4, 5. 0·01 unit oxytocin + 0·02 mg. trihydroxyœstrin in 0·1 c.c. alcohol. (*b*) 1. 0·02 unit oxytocin + 0·02 mg. trihydroxyœstrin in 0·1 c.c. alcohol. 2. 0·014 unit oxytocin + same amount of œstrin. 3. 0·01 unit oxytocin + 0·1 c.c. absolute alcohol. The height of 3 falls between 1 and 2, so that 0·02 mg. trihydroxyœstrin "neutralizes" approximately 0·007 unit oxytocin.

Guinea-pig, 540 g.; oöphorectomized 12 weeks previously. (*c*) 1, 2, 3, 6. 0·01 unit oxytocin + 1 c.c. distilled water. 4, 5. 0·01 unit oxytocin + 1 c.c. saturated aqueous solution of trihydroxyœstrin (1 c.c. contains approx. 0·02 mg.). Interval between doses: (*a*) and (*b*) 25 min., (*c*) 10 min.

The mature uterus.

Normal behaviour. Two types of activity *in vitro* are found, and these are illustrated in Fig. 6. The first is quite irregular in character, and is not easily described. The second approaches a regular succession of maximal contractions, and the tracing shows a fairly steady and well-marked base line. In a batch of large guinea-pigs (about 500–600 g.), probably parous, the tracings from animals during œstrus and diœstrus were compared, but the type of tracing obtained could not be correlated with the absence or presence of œstrus (Fig. 8). Many of the tracings approximated to the second type, irrespective of the condition of the animal from which the uteri were taken. After the muscle had been in the bath for an

hour or two, all types of tracing tended to settle down to the second type. In comparative experiments, therefore, it is probably only legitimate to use the first hour and a half of the tracing.

The effect of increasing the temperature is to increase the frequency of the contractions (Fig. 6); their amplitude varies with the length of the strip, and therefore with the size of the uterus if the whole horn is taken. For these reasons more stress is laid upon the form of the contractions than upon their frequency and amplitude. The two horns from the same

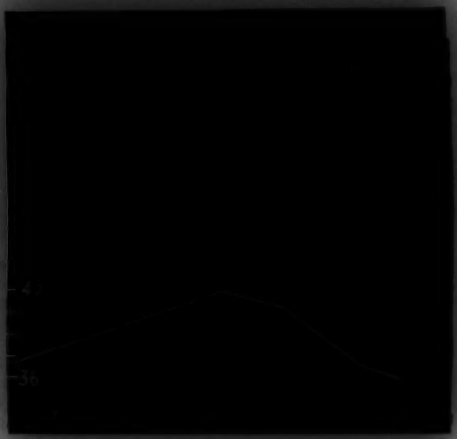

Fig. 6. Typical tracings from the uteri of two adult guinea-pigs, 550 and 520 g. respectively. The lower tracing shows the effect of temperature change upon the contractions (scale and continuous line). $T = 15$ min.

uterus give as a rule the same type of contraction, although one or two exceptions to this have been encountered; no error from this source is likely to occur if sufficient experiments are done.

Effect of œstrin. (*a*) Some of the saline solution was saturated with pure crystalline ketohydroxyœstrin (probably between 0·2 and 0·4 mg./100 c.c.). One horn of the uterus was then put up in this solution, and the other in the original saline solution. There was not sufficient difference between the form of contraction in the two cases to warrant any statement as to the action of œstrin given in this manner.

(*b*) Crystalline ketohydroxyœstrin was dissolved in absolute alcohol, 2 mg./100 c.c., and each horn of the uterus suspended in the ordinary way

in the saline solution. After the tracing had become established 0·1 c.c. of the œstrin solution was added to one bath and 0·1 c.c. of absolute alcohol to the other. In one experiment this procedure was repeated twice, so that finally three times the usual quantities of œstrin solution and alcohol respectively were present. In neither horn was the tracing appreciably altered by this treatment, and this was the rule in these experiments.

Fig. 7. (*a*) Tracings from the two horns of an adult guinea-pig before (above) and after (below) œstrin injection respectively (see text). (*b*) Corresponding tracings from the two horns of the least satisfactory control guinea-pig, in which the second tracing most nearly resembled the tracings after œstrin injection. (The animal was on œstrus when the upper record was taken.)

(*c*) Two guinea-pigs of approximately equal age and weight (300–350 g.) were anæsthetized with ether, one uterine horn removed aseptically from each, and tracings from these horns taken in the usual way. The day after the operation a course of subcutaneous injections was begun; one animal receiving œstrin and the other the solvent (10 p.c. alcohol). The injections were given hourly (except through the night), and continued for 48 hours; this represented about 25 c.c. of fluid, and 0·6 mg. of œstrin. 18–24 hours after the last dose the animals were killed, the

remaining horns removed, and their contractions recorded. The types of contraction obtained from the horns of the control guinea-pig agreed fairly well, in spite of the interval between the records. In the other animal, however, the effect of the œstrin was distinctly seen (Fig. 7). The contractions recorded from the second horn were relatively infrequent, regular, and apparently maximal; the muscle was fully relaxed between them, and this, together with the absence of minor contractions, gave a very definite base line to the tracing. This result was invariable.

Discussion.

It is clear that the injection of œstrin does modify the *in vitro* contractions of the uterus, and it would be expected that the same effect would be apparent when œstrin is produced by the animal itself, *i.e.* during œstrus. One has not been able to demonstrate this for the guinea-pig, possibly because of the age and uncertain history of the animals used. It has been stated, however [Frank, Bonham and Gustavson, 1925; Durrant and Rosenfeld, 1931; Harne, 1931], that in the rat the type of activity exhibited by the excised uterus during œstrus is similar to that just described after injections of œstrin into the guinea-pig. Reynolds [1931], recording from a fistula, has found that the uterus of the rabbit is inactive during dioestrus and after oöphorectomy, but active during œstrus and after injection of œstrin. This agrees with the present findings in oöphorectomized guinea-pigs (although these require confirmation, as previously pointed out), but it would be ambiguous to say whether normal guinea-pig uteri are more or less active after œstrin injection; the activity seems rather to change qualitatively and become more coordinated. Azuma and Soskin [1932], using a fistula, found uterine motility to be diminished in the dog 24 hours after œstrin injection.

The uterus of the pregnant animal.

Normal behaviour. In the pregnant guinea-pig only one horn of the uterus is usually involved; this is referred to as the "pregnant horn," and the other as the "non-pregnant horn." The former is enormously hypertrophied, and its wall is so thin as to be transparent. In the majority of the experiments only one fœtus has been found: this attains at term a length of about 11 cm. measured from the frontal bone to the posterior tip of the spinal column. In the absence of any knowledge of the previous history of the animals, the length of the fœtus has been used as a guide to

the stage of pregnancy. On several occasions two fœtuses have been found, but the behaviour of the uterus on these occasions has been similar to that observed when the fœtus has been a single one of the same length as each of the pair. The non-pregnant horn is larger than that of a non-pregnant animal, and is occluded at the lower end by a plug of mucus.

Longitudinal strips cut from the pregnant horn give in the early stage of pregnancy a series of spontaneous contractions which are fairly regular and maximal, with a well-marked base line between. Later in pregnancy

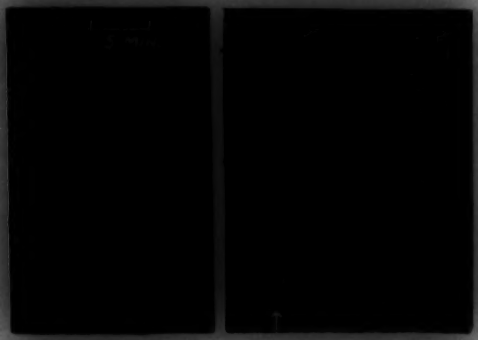

Fig. 8. Pregnant guinea-pig, fœtus (one) 7 cm. Longitudinal strip from pregnant horn, showing rapid relaxation in bath. The gap in the tracing represents 50 min., and during this time no spontaneous contractions took place. At the arrow, 0·01 unit oxytocin was added to the bath (100 c.c.).

these contractions become less frequent as the record progresses, and may cease altogether. Later still in pregnancy the strip rapidly relaxes on being placed in the bath, and gives no spontaneous contractions whatever; the tracing appears like a line drawn with a ruler (Fig. 8). It is not yet clear whether spontaneity returns at term; the whole series of experiments is being repeated with animals whose stage of pregnancy is accurately known. Throughout pregnancy the non-pregnant horn gives maximal, fairly regular spontaneous contractions.

Action of oxytocin. This has been investigated only on the inactive strips of the uterus of late pregnancy (fœtus 9–10 cm.). When oxytocin is added to the bath in which these are suspended, they break into perfectly regular, smooth, maximal contractions (Figs. 8 and 11), which apparently

continue so long as the oxytocin is left in the bath. The concentration of oxytocin necessary to bring these about is of the same order as that usually required to produce a nearly maximal contraction of the virgin uterus, *i.e.* 0·01–0·02 unit in 100 c.c. The exact threshold dose for an individual strip depends in part upon the load carried by the writing lever, *i.e.* upon the tension of the muscle, as the following experiment shows. If a very small dose of oxytocin be added to the bath the tracing continues unaltered as a straight line: if the dose be increased a point is reached at which the base line begins to rise slowly. This rise continues for 2 or 3 min., when suddenly a maximal contraction occurs, with the usual almost instantaneous rise of the lever; after the contraction the

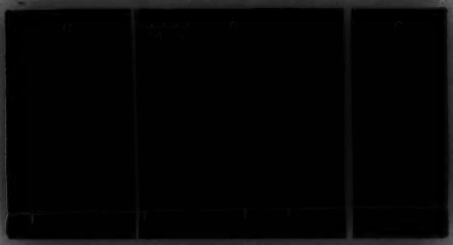

Fig. 9. Pregnant guinea-pig. Length of fœtus (one) 9 cm. No spontaneous contractions. (*a*) Effect of 0·015 unit oxytocin. (*b*) Later, after removal of load from lever so that muscle was just balanced. Signals: (1) 0·02 unit oxytocin; (2) same; (3) load applied to lever. (*c*) Contraction 15 min. later; similar contractions followed regularly at 15 min. intervals.

lever returns to the original base line, and there is a relatively long interval before the next contraction, which is also preceded by the slow shortening. A further increase in the amount of oxytocin present yields the usual series of contractions, which are more frequent and without, or almost without, the preliminary shortening. If now the load on the lever be removed, so that its weight is just sufficient to balance the strip, it is found that what was before an adequate dose of oxytocin, now gives rise only to an exaggerated version of the slow type of contraction, and although this may produce considerable shortening of the strip, there may be no sign of the usual brisk maximal contraction and relaxation. Application of a load to the lever, however, immediately initiates such a contraction, and further uncomplicated contractions occur at regular intervals (Fig. 9).

The importance of tension is shown also by Fig. 10, which shows that the frequency of contraction varies directly as the load on the lever. The rhythm also varies with the temperature of the bath.

Fig. 10. Pregnant guinea-pig; length of fœtus (one) 10 cm. Longitudinal strip from pregnant horn. Tracing after 0·03 unit oxytocin, with lever just balanced. Arrows indicate application or removal of load, as follows: (1) +0·33 g.; (2) +0·48 g.; (3) +0·60 g.; (4) −0·60 g.; (5) −0·48 g.; (6) −0·33 gm.; (7) +1·41 g.

Fig. 11. Pregnant guinea-pig. Length of fœtuses (two) 9 cm. Strip of pregnant horn in 100 c.c. bath. At signals: (1) 0·025 unit oxytocin; (2) 0·2 mg. ketohydroxyœstrin in 1 c.c. *N*/10 NaOH + exactly equivalent amount of HCl; (3) fresh solution in bath; (4) 0·025 unit oxytocin; (5) 1 c.c. *N*/10 NaOH + exactly equivalent amount of HCl.

An excessive dose of oxytocin tends not only to initiate contractions, but also raises the base line between these, so that the uterus is in a state of continued partial contraction.

Action of œstrin. If a large amount of oxytocin has been used, so that rhythmic contractions are superimposed on a permanent tonus, the addition of œstrin to the bath reduces this tonus, and full relaxation

occurs between the beats. In such circumstances the rhythm may be accelerated [Marrian and Newton, 1932]; what happens when still more œstrin is added cannot be determined, owing to the difficulty of getting large amounts into solution. If just sufficient oxytocin to give a series of rhythmic contractions, with no general rise of base line, is present, then, on treatment with œstrin, the contractions are diminished in frequency and amplitude. In other words, the effect of œstrin is to neutralize that of oxytocin. These effects have been obtained using alcoholic and alkaline solutions of œstrin, and the action of the solvent has been controlled (Fig. 11).

General discussion.

There seems to be no doubt that the muscular activity of the uterus may be modified by injecting preparations of œstrin into animals. Some of the work already quoted suggests that œstrin stimulates, and some that it depresses the uterus, but results from the same animal appear consistent. There is probably, therefore, a species difference, but this cannot with confidence be stated until it is established that the uterus as studied by fistula in the intact animal and the excised uterus behave in the same way at the same time.

The fact that the effect of œstrin injection can so easily be traced in the activity of the excised uterus points to some stable alteration having taken place in the muscle. The most obvious change of this sort is an enlargement of the uterus, and this may be associated with increase in size, number, or both, of the muscle fibres. Andrei [1924] has reported hyperplasia in the œstrous rabbit, and Anopolsky [1928] increase in length of the tubal muscle fibres of the sow during heat. Before attributing a "pharmacodynamic" action to œstrin [Brouha and Simmonet, 1927], it is important to decide whether such structural alterations in the muscle would be sufficient to account for a qualitative change in activity such as is seen in the guinea-pig. At first sight the correlation between activity and size, as seen in virgin, mature and involuted uteri, seems straightforwardly quantitative; but the large uteri of large guinea-pigs frequently yield tracings suggestive of those obtained after œstrin injection, and the non-pregnant horn of the pregnant animal, which is considerably enlarged, certainly gives this kind of tracing. Further, if the characteristics of this type of activity (diminished frequency of contraction, the maximal nature of such contractions as do occur, and the tendency to complete relaxation between the beats) were exaggerated and pushed to the limit, the same stages would be anticipated as do actually

occur with progressive hypertrophy in the pregnant horn of the pregnant animal. The dissimilarity between the activity of the pregnant and non-pregnant horns (apparently overlooked by Knaus [1927], who used the latter as a criterion for the former) emphasizes the importance of structural or mechanical conditions as distinct from hormonal factors. There is at present insufficient evidence to decide whether the change in size of the uterus after œstrin injection has anything to do with the coincident change in its activity, but this possibility cannot be ignored.

In the experiments where œstrin is added to the muscle bath, instead of being injected previously into the animal, the most striking phenomenon is its antagonism to oxytocin, but it is not legitimate to assume that this is physiological. There seems to be no conclusive evidence as to the normal interaction between these two hormones. Parkes [1930] produced immediate abortion in a certain percentage of mice by following up 12 hours of œstrin injection with the injection of 1 unit of pitocin, to which the mice were normally insensitive. He argued from this that there was some immediate action on the muscle, sensitizing it to pitocin; this action may not have been due to œstrin, however, since no crystalline preparation was available. Moreover, Illingworth, Marshall and Robson [1932] and Jeffcoate [1932] claim that there is a substance sensitizing the uterus to pitocin in extracts of urine free from the known sex hormones. Brouha and Simmonet [1927] describe the types of contraction produced in the isolated uteri of guinea-pigs by pituitrin, when these are normal when killed, or previously treated with follicular extracts. After œstrin, instead of a tonic contraction, a series of rhythmic contractions are obtained; this agrees with our findings, but does not throw light on the sensitivity of the uterus. Pompen [1933], however, taking records from the living animal by means of Trendelenburg's method [1913], definitely states that œstrin injection lowers the threshold to pituitrin in rabbits, cats, and guinea-pigs.

Summary and conclusions.

1. The types of uterine contraction met with in the isolated uteri of guinea-pigs, immature, mature, pregnant and oöphorectomized, are described, together with certain temperature and tension effects.

2. Œstrin, *in vitro*, has no certain action upon the immature or mature uterus, but œstrin previously injected into the intact adult animal profoundly modifies the activity of the uterus subsequently excised. The possible relation of this change in activity to hypertrophy of the muscle is discussed.

3. The action of oxytocin on the immature, the pregnant, and involuted uterus is described, and it is shown that this action is in each case partially neutralized by the simultaneous presence of œstrin in the muscle bath. This effect of œstrin is not necessarily related to its true physiological action.

4. The uteri of guinea-pigs 3–4 months after oöphorectomy can be used for the assay of the oxytocic pituitary principle, but the method is tedious and probably only of value for confirmatory purposes.

I wish to thank Dr G. F. Marrian, who, by providing crystalline œstrin and by much valuable assistance, has made this work possible.

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THE EFFECT OF THE CARDIAC CONTRACTION UPON THE CORONARY FLOW.

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In spite of the extensive researches made during recent years, some of the most fundamental questions concerning the coronary circulation still remain a field of considerable debate. The question whether the blood supply to the heart muscle is maximal during the period of systole or during the period of diastole seems to be the centre of most violent and uncompromising exchanges of opinion.

A comprehensive review of the literature on the coronary circulation was recently made by Condorelli [1932]. Amongst other problems, the author deals in a purely objective and unbiased way with the different opinions about the extent to which the cardiac contraction may affect the coronary flow. Condorelli concludes that the blood supply to the left ventricle during the period of systole is but little affected by the systolic rise of the aortic blood-pressure. A second monograph on the same subject by Hochrein [1932] is chiefly devoted to a restatement of the author's own opinions and to an attempt to prove that the maximal blood supply to the heart occurs, as in any other organ, during the period of systole. This conclusion is supported by references to the author's former publications. No new facts are provided, and the criticism of his experiments which was made in communications from this laboratory [Anrep, Davis and Volhard, 1931; Davis, Littler and Volhard, 1931] is left unanswered although it is mentioned. We refrain from any polemical discussion of this problem, as we prefer to subject the question to a still further experimental test.

If the coronary artery were suddenly and completely blocked for a short time during some definite period of the cardiac cycle, the effect of this block on the blood supply to the heart muscle would depend on whether the clamping period coincided with the period of maximal or of

minimal blood flow through the artery. If the blood flow through the coronary artery were entirely uniform throughout the cardiac cycle, repeated short periods of clamping would always have the same effect upon the blood flow, and it would be immaterial whether these periods of clamping fell within the systole or the diastole. So long as the blood flow through the coronary artery is not uniform, however, and so long as periods of larger and smaller flows stand in some definite relation to the cardiac cycle, clamping of the coronary artery which coincided with the periods of reduced flow would produce a small diminution of the flow through the artery, while clamping which coincided with periods of large flow would produce a large diminution. If at some definite phase of the cardiac cycle the flow is at a standstill, clamping of the artery which exactly coincided with this phase should produce no change in the blood flow whatever. By gradually shifting the period of clamping so as to make it fall within different stages of the cardiac cycle, it should be possible to determine at which stage the clamping produces the minimal effect and thus to decide whether the blood supply to the heart is smaller during systole or during diastole.

If a glass tube is provided at each end with a rotating tap, the periods of closing and opening of which could be made to coincide or to alternate, the effect of closing these taps upon the flow of liquid through the tube would be maximal in the case of alternate closing; and if the closure of one tap were to coincide exactly with the opening of the other, the flow would be reduced to zero. Approximately similar conditions should hold for the heart, except that in this case we deal not with a rigid tube but with blood vessels which have certain elastic properties. In this respect one could compare the effect with that produced by turning two taps which are placed at the ends of a rubber tube. In the case of alternate closure of the taps, the rubber tube would be distended when the distal tap was closed and the proximal tap opened. When the proximal tap was closed and the distal one was opened, the rubber tube would empty itself to an extent determined by its elasticity. Therefore the flow of liquid would be considerably reduced but not completely stopped.

A constant effect of periodical clamping of an artery can only be hoped for if the clamping always takes place during some definite period of the cardiac cycle, and if the length of each clamping period is maintained constant. The most definite results should be expected if the length of clamping is made equal to the length of the period of the minimal or of the maximal blood flow through the artery. The apparatus necessary for this purpose should therefore be constructed so as to allow the clamping

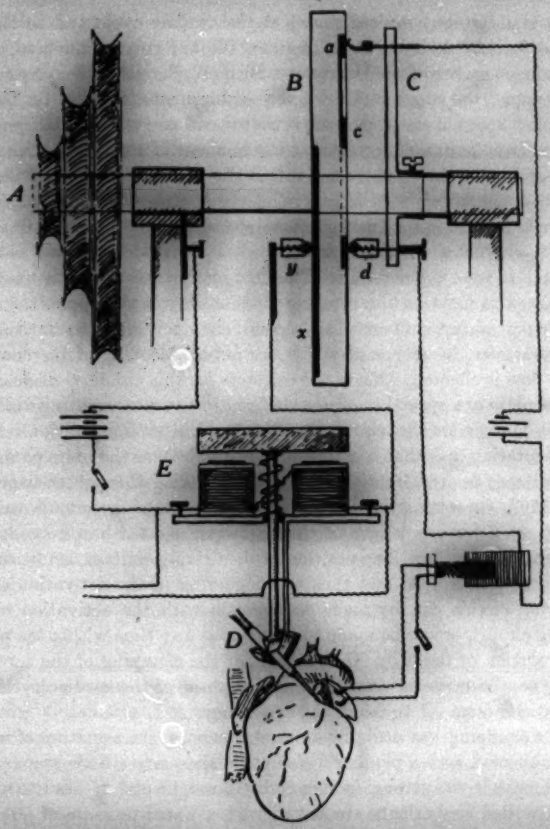

Fig. 1. The clamping apparatus. *A* are pulleys for driving the apparatus. *B* is an ebonite disk carrying a spring contact *a* for the "driving circuit" and a brass plate contact *x* for the "clamping circuit." The arm *C* is stationary and is insulated from the shaft; it can be fixed in any position in relation to the rotating disk, so that the moment at which the single induction shocks affect the heart can be changed; *c* is a brass circle which is in constant contact with the spring ball *d*. The brass plate *x* forms a sector of 180° of the disk close to the shaft and 36° at the periphery; the spring ball contact *y* is fixed on a swing arm which can be adjusted in any desired position so as to activate the clamping circuit for any duration between 0·1 and 0·5 of a revolution of the disk. *E* is the electromagnet and *D* the plunger-like extension of its armature which clamps a short piece of rubber tubing connected with the coronary cannula.

to be made at any desired phase of the cardiac cycle and to be continued for any desired length of time. We are greatly indebted to the instrument maker of the University, Mr F. W. King, for the construction of an apparatus which fulfilled these requirements (see Fig. 1). The important feature of the apparatus is that it can activate two independent electric circuits in such a way that the moment of activation of either of them and the duration of the activation of one can be altered at will. One circuit is connected with a pair of electrodes placed on the auricle or ventricle of the heart. Through these electrodes, by means of single induction shocks, the heart is driven to beat at a constant rate between 100 and 160 beats per minute. The contact for this circuit is made between a wire fixed on the rotating ebonite disk of the apparatus and a stationary spring contact. The second circuit is used to activate an electromagnet, by means of which the actual blocking of the coronary blood flow is effected. One of the contacts for this circuit is made out of a brass plate of a special shape and is fixed to the same rotating disk. The second contact for this circuit can be placed in such a position relative to the rotating disk that it can be made to slide over the brass plate contact for any desired fraction of the disk, cutting through sectors from 36 to 180°. On setting the disk in rotation, the clamping circuit can thus be activated for any length of time between one-tenth and one-half of the period of rotation. The position of the driving contact can be shifted round the whole disk, and thus the beginning of the activation of the clamping circuit can be made to coincide with the activation of the driving circuit, or can be made to follow it at any time within the period of revolution of the disk. In this manner the clamping of the coronary artery can be made to occur during any phase of the cardiac cycle and to continue from 0·1 to 0·5 of the cycle length.

The clamping was made by an electromagnet, the armature of which was connected with a plunger which could slide into a little groove. The electromagnet was strong enough to lift a weight of 2 kg. and to clamp a thin-walled rubber tube connected with a water pressure of 500 mm. Hg. The excursions of the electromagnet did not exceed 4 mm. and the clamping was extremely rapid. The registration of the clamping was made by means of a small electromagnetic signal connected with the clamping circuit. In order to determine the rapidity of the clamping and the accuracy of its registration by the signal, the following tests were made. A reservoir containing water was placed at a suitable height and connected by means of a metal tube to a cannula, the nozzle of which was provided with a short piece of thin rubber tubing. This rubber tube was

placed in the groove under the plunger of the electromagnet, and the flow of water through the tube was measured before and during periods of clamping. The disk bearing the contact was set in rotation at some definite rate, and the length of each individual clamping of the tube was changed between the measurements of the flow by one-tenth of a revolution of the apparatus. Even under the best conditions one would hardly expect that, with each prolongation of the clamping time by one-tenth, the flow would diminish by exactly 10 p.c. The apparatus, however, worked quite satisfactorily, as can be seen from the following example. The free flow of water through the tube was 55 c.c. per min. The clamping contact was set in rotation at 150 revolutions per min., and the length of each clamping was increased by one-tenth of a revolution from 0·1 to 0·5, an increase of 0·04 sec. each time. The corresponding diminutions of the flow with each increase of the length of clamping were 9·0, 19·0, 29·0, 39·5 and 49·0 p.c.

Hot-wire registration of the outflow of water from the reservoir, or of the outflow from the cannula beyond the place of clamping, showed that the clamping was extremely rapid, the flow being arrested within 0·02 sec. The signal which was used for the registration of the clamping was found to work quite synchronously with the electromagnet. This was ascertained by simultaneously photographing on a roll-paper camera the movement of the armature of the electromagnet and the movement of the signal.

The blood flow through the coronary artery.

For the success of the experiments, it was imperative to have an accurate measurement of the minute flow through the coronary artery, and an exact knowledge of the phase of the cardiac cycle during which the clamping of the blood supply took place. The cardiac cycle was registered by means of a Wiggers optical manometer (vibration frequency 130–150 per sec.) which was inserted through the left subclavian artery into the aorta, when the experiment was made on the whole animal. In some experiments this was supplemented by an electrocardiogram, for which direct leads were taken from the ventricle. All our observations were made on the circumflex branch of the left coronary artery. The experiments were made on dogs weighing 8–12 kg., anæsthetized with chloralose or sodium luminal. In the whole animal, heparine was used to prevent coagulation. The chest of the experimental animal was opened through a midsternal incision, and artificial respiration was carried out by an

"Ideal" respiratory pump. The coronary artery was either perfused from a reservoir containing blood, which was placed at a suitable height above the animal, or from the aorta of the animal through a connection leading from the subclavian artery to a cannula which was inserted into the peripheral end of the coronary artery. The perfusion from the reservoir (constant pressure perfusion) could be switched over to the perfusion from the aorta (autoperfusion with the pulsatile pressure) by turning a tap with a large bore (Fig. 2). All connections between the perfusion source, whether aorta or reservoir, and the cannula were rigid. The few end-to-end glass connections were made with very inelastic thick pressure tubing. These precautions were especially necessary in the case of the autoperfusion, since otherwise the transmission of the pulse pressure from the aorta to the coronary artery might have been delayed and the cardiac systole would not have coincided with the rise of the blood-pressure in the artery. In consequence of this, the maximal pressure in the coronary artery would have been shifted somewhat towards the diastole. On many occasions we measured simultaneously the blood-pressure in the aorta and in the coronary cannula, and proved to our satisfaction that the rigidity of the apparatus was sufficient to prevent such a displacement. The blood flow through the artery was recorded by means of a small Stolnicov stromuhr of about 10 c.c. capacity. The time taken for a flow of 5 c.c. was measured with an accuracy of half a second, which means that with an average blood flow of 20–25 c.c. per min. the measurements were accurate to about 0·2 c.c.

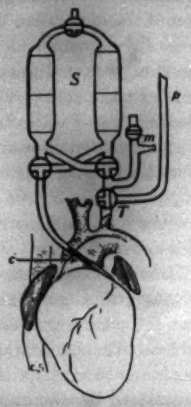

Fig. 2 shows the arrangement of the apparatus. p is the connection with the perfusion reservoir containing blood, m is the optical manometer, S the stromuhr, c the place of clamping by the electromagnet, and T is a tap by means of which the perfusion of the coronary artery with the constant pressure can be changed to the autoperfusion with the pulsatile aortic pressure. The apparatus is rigid and its taps have a bore of 4 mm. in diameter.

On the way to the coronary artery, and immediately preceding the cannula which was introduced into it, the blood was made to pass through a short piece of thin-walled rubber tubing, the internal diameter of which was 3 mm., while the length of the free part of the rubber tube was equal to 3–4 mm. The rubber tube was placed in a groove underneath the

plunger of the electromagnet which, when periodically activated, clamped the tube in the middle of its free part. The plunger was 2 mm. wide, so that the displacement of fluid in the tube during the clamping was extremely small (about 0·01 c.c.).

Fig. 3. A and B show the registration of the exact moment and the duration of the coronary clamping in relation to the cardiac cycle. Records of the aortic blood-pressure; the top line is the signal recording the clamping by an upward movement. In A the clamping starts during systole and extends slightly into the diastole; in B it starts in diastole and ends soon after the beginning of systole. Records C and D show a similar experiment, together with a hot-wire registration of the inflow of blood into the perfused coronary artery. The interrupted straight line across the records is the base line for the hot wire. With increase of the blood flow, the shadow of the string moves downwards. The vibrations of the hot-wire record are caused by the impacts of the electromagnet. For other explanations see text. All the tracings in this paper should be read from left to right. Time in 0·2 sec. The time lines in these tracings run somewhat obliquely because of an instrumental error; they are nevertheless accurate.

The heart was driven to beat at a constant rate throughout a whole experiment. Several consecutive measurements of the free blood flow through the coronary artery were made. The clamping circuit was then switched in, and the blood-flow measurements were repeated. In order to know the exact time within the cardiac cycle during which the clamping

took place, the aortic blood-pressure was recorded photographically, together with the movement of the electromagnetic signal. As said before, in some experiments this was supplemented by an electrocardiogram. An example of an actual record is given in Fig. 3A and B. In the first the clamping starts during systole and extends somewhat into the diastole. In the second it starts during diastole and extends into the next systole. The relative position of the clamping period within the cycle was then changed by adjusting the moment at which the driving induction shock was sent into the heart. Measurements of the blood flow were repeated with every new change of the clamping. In between each change the clamping was stopped, and the free flow through the coronary artery was again determined. Usually the procedure was such that three or four measurements of the free flow were interimposed between two or three measurements during the periods of clamping. In some experiments with perfusion of the artery with a constant pressure, a hot-wire registration of the inflow of blood into the artery was also made. An example of such a record is given in Fig. 3C and D, which was obtained from an experiment on the whole animal. In the first record the clamping starts a little after the onset of systole and extends slightly into the diastole. In the second it starts at the dicrotic notch and continues during a long stretch of diastole. The hot-wire record, although distorted by the vibrations caused by the electromagnet, shows that the systolic diminution of the blood flow develops considerably more rapidly in Fig. 3C than in Fig. 3D. In the first case the diminution is started by the systole and accentuated by the clamping of the artery. In the second the diminution is due to the systole alone; the clamping begins at the moment of the minimal blood flow and only serves to prolong this diminution.

Hochrein states that in the whole animal, when the coronary artery is perfused at a constant pressure, the maximal blood flow occurs during systole, which would imply that during systole the heart muscle opposes the minimal resistance to the blood flow. Since, during normal pulsatile aortic perfusion of the artery, the maximal pressure in the coronary artery is synchronous with the maximal pressure in the aorta, one would expect in the whole animal a most vigorous systolic perfusion of the heart. This is the reverse of what we find in the heart-lung preparation [Anrep and Häusler, 1928, 1929] and in the isolated perfused heart [Rössler and Pascual, 1932]. But Hochrein considers that the conditions of the circulation in the heart-lung preparation are so abnormal that the coronary circulation may be entirely changed. Without going into this question any further, we should like to say that it is difficult to under-

stand why a coronary artery, which is perfused at a constant pressure from an outside source, should behave in such a strikingly different manner in the heart-lung preparation and in the whole animal. In our previous experiments we found no such difference. Whether in the heart-lung preparation or in the isolated heart or in the whole animal, the perfused coronary artery had invariably a minimal blood flow during systole

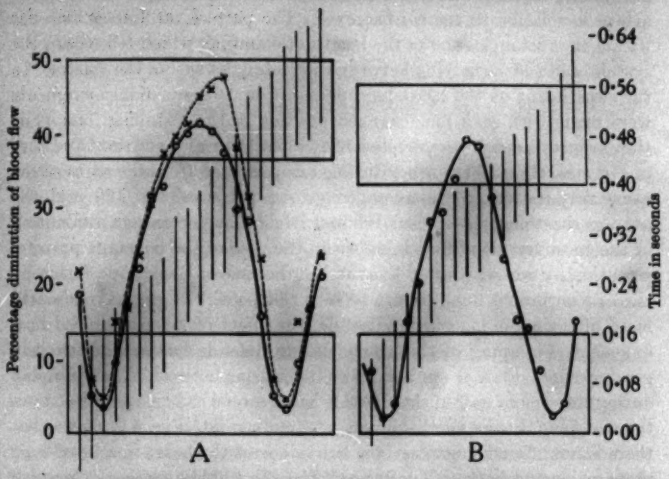

Fig. 4. Percentage diminution of the minute blood flow through the left circumflex coronary artery during short periodical clampings of the artery at various phases of the cardiac cycle. The diagram should be read from below upwards. The two rectangles at the bottom and at the top represent the duration of the systole, and the space between them represents the duration of the diastole. The ascending row of straight lines shows the duration of the clamping and its position within the cycle. A is from an experiment on the heart-lung preparation; B is from an experiment on the whole animal. The dotted line with crosses shows the result of clamping during a constant pressure perfusion; the continuous line with circles shows the result during autoperfusion.

and a maximal during the second half of diastole. However, since the objection has been made, we decided to perform our experiments on the whole animal and to compare the results with those obtained on the heart-lung preparation. The result of two experiments is given in Fig. 4. The left-hand side of the figure shows the effect of clamping of the coronary artery at different periods of the cardiac cycle in a heart-lung

preparation, the right-hand side shows the results obtained on the whole animal.

The figure should be read from below upwards. The width of the two rectangles at the bottom and at the top of each side of the figure represents the average length of systole, while the distance between the rectangles represents the length of diastole. The gradually ascending row of straight lines shows the duration and the position of the clamping of the coronary artery in relation to the cardiac cycle. The part of the lines which lies within the rectangles shows the length of clamping which fell within the systole, and the parts lying between the rectangles within the diastole. In the experiment on the heart-lung preparation, two sets of measurements were made with each new changed position of the clamping, one while the coronary artery was perfused from a reservoir at a constant pressure of 110 mm. Hg, and the other during autoperfusion from the aorta of the same preparation. (The average systolic pressure was 160 and the average diastolic pressure was 100 mm. Hg.) The percentage diminution of the coronary blood flow is shown in the case of the constant pressure perfusion by crosses (dotted line) and in the case of the autoperfusion by circles (continuous line). It can be seen that, with the gradual encroachment of the clamping on the diastole, the effect of it on the blood flow increases. Clamping of the artery during diastole has a conspicuously greater effect than a similar clamping during systole. The clamping during the second half of the diastole has a somewhat greater effect than the clamping during the beginning of the diastole. Fig. 4B shows that there is no difference between the behaviour of the heart in a heart-lung preparation and in the whole animal. The effect of the coronary clamping was found to be in both cases the same in character and magnitude.

In some experiments on the whole animal the clamping of the coronary blood flow at various periods of the cardiac cycle was made during constant pressure perfusion as well as during autoperfusion from the aorta. In other words, the experiment was done exactly in the same way as the one on the heart-lung preparation, the results of which are given in Fig. 4A. The results were the same in the two cases. A large number of observations was made by us on many animals and all cases gave the same results, except that the extent of the effect differed from one animal to another. The greatest diminution of the coronary blood flow during diastolic clamping, which occupied about one-half of the diastole, was 54 p.c. The smallest effect was 28 p.c. Systolic clamping had in some experiments almost no effect on the coronary blood flow, and in others it slightly reduced the flow, usually by not more than about 5 p.c.; only

in one experiment was the diminution of the flow as great as 10 p.c. We found no difference in the result after section of the cervical vagi. From a consideration of these experiments the conclusion seems to be unavoidable that the blood flow through the coronary artery is maximal during diastole and minimal during systole. It also seems obvious that in this respect there is no fundamental difference between the heart-lung preparation and the whole animal and that, disregarding minor points, the circulatory conditions in the perfused and in the autoperfused artery are comparable.

The blood-pressure in the coronary artery.

Further evidence in favour of this conclusion was obtained by measurements of the blood-pressure in the coronary artery between the place of clamping and the heart. If, during the periods of the systolic clamping, there is little or no inflow of blood into the heart, then the pressure in the cannula, from the beginning of the clamping until the time when the next diastole sets in, must remain approximately at the level at which it was during the moment of clamping. On the other hand, if during the diastolic clamping there is no restriction of the blood flow into the heart muscle, the pressure in the coronary cannula should progressively fall during the whole period of clamping. In order to test this, the coronary cannula was provided with a wide side branch, which was connected to a high-frequency optical manometer. The stromuhr was omitted, so that the whole preparation consisted in the establishment of a communication between the aorta and the coronary cannula. This connection was made entirely rigid except for the 3–4 mm. length of the rubber tube under the plunger of the electromagnet. Thus during the clamping the pressure was measured in the peripheral end of the coronary artery. The cannula carrying the side tube was introduced, as in the blood-flow experiments, into the circumflex branch of the left coronary artery. The heart was driven at a constant rate and the blood-pressure in the artery was recorded during the periods of clamping at various phases of the cardiac cycle. Fig. 5 shows the effect of a purely systolic and of a purely diastolic clamping. The records speak for themselves. During the systolic clamping, not only is there no fall but there is a definite rise of the pressure in the artery; during the diastolic clamping there is a precipitate and conspicuous fall. This effect is equally well marked in an artery which is perfused at a constant pressure and in one which is perfused by the pulsatile aortic pressure. The rise of pressure during systolic clamping

was not observed in all experiments. In many cases, instead of rising, the pressure remained steady at the level at which it was when the clamping took place. It fell abruptly only with the onset of the diastole. In other experiments there was a small fall of pressure during the systolic clamping. This fall, however, was negligible as compared with the huge drop of pressure observed during the diastolic clamping, when the pressure

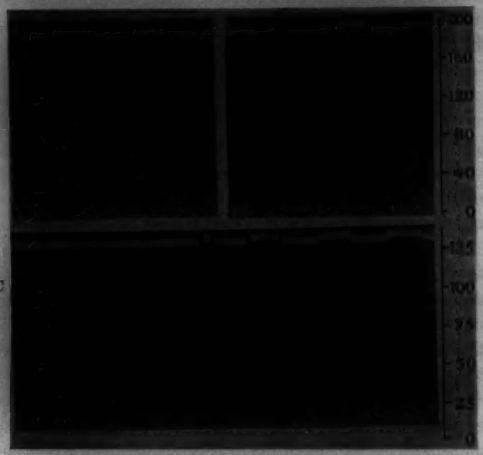

Fig. 5. The effect of clamping of the coronary artery upon the coronary blood-pressure. The top line is the signal recording the clamping; the dotted curves show the blood-pressure before the periodical clamping was begun. Tracings A and B are taken from the same experiment. In A, systolic clamping, in B, diastolic clamping. Tracing C, from another experiment, shows the beginning of a clamping period. The first clamping is very short and purely diastolic. The next clampings start during systole and involve a part of the diastole. Note that the drop of the blood-pressure occurs only during the diastolic part of the clamping. During the systolic clamping the blood-pressure in these two experiments shows a considerable rise.

may fall almost to zero. Only in one experiment was the fall of pressure during the systolic clamping rather considerable, but even here it was still very much smaller than that taking place during the diastolic clamping. In this case a particularly large auricular branch was found to leave the coronary artery just below the nozzle of the cannula.

The difference between the effect of systolic and diastolic clamping is even better observed when the driving of the heart is omitted. The heart

is allowed to beat at its own rhythm, while the clamping is set to work at a rhythm which is slightly faster or slightly slower than the heart rate. The clamping thus gradually shifts with each heart beat from diastole more and more into the systole and then again into the diastole. In this

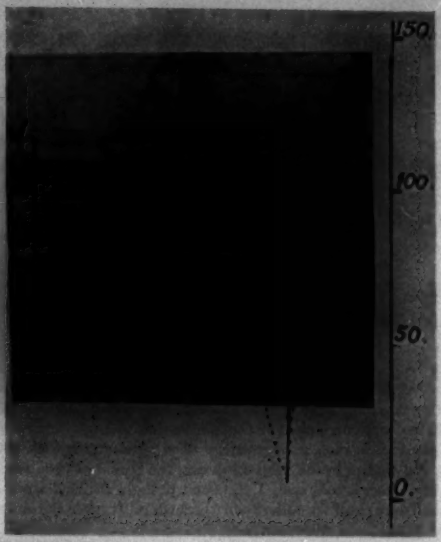

Fig. 6. Whole animal. The effect of two successive clampings of the coronary artery upon the coronary blood-pressure. The dotted curve is a retraced record of the aortic blood-pressure. (1) is the end of a clamping which was partly systolic and partly diastolic, (2) a systolic clamping, (3) a diastolic clamping. The dotted line continuing the drop of pressure in (3) is retraced from the 21st cycle following, in which an exactly similar relative position of the clamping took place and in which the camera was moved so as to include the low pressure levels. An E.C.G. (direct leads from the ventricle) was taken during this experiment. During the systolic clamping the blood-pressure fell by 16 mm. Hg; during the diastolic clamping it fell by 94 mm. Hg, almost reaching zero.

way one gets all the possible combinations in the time relations between the cardiac cycle and the clamping. It would be impossible to reproduce an original record of such an experiment on account of its length. Therefore we give only a small section of a record (see Fig. 6), in which a systolic clamping is followed by a diastolic clamping, and a diagram which is

composed of retraced pressure records obtained in an experiment with autoperfusion on the whole animal (see Fig. 7). We would like again to stress the fact that experiments on the whole animal and on the heart-lung preparation with autoperfusion and with constant pressure perfusion gave the same results.

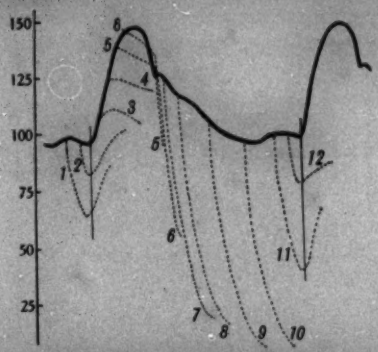

Fig. 7. Retraced superimposed records of changes in the blood-pressure in the coronary artery during its clamping at various phases of the cardiac cycle. All the records were obtained in the same experiment on the whole animal. The continuous curve represents the blood-pressure in the coronary artery when no clamping took place. The dotted lines show the change in the pressure during the period of clamping. The length of clamping in all cases is the same. 1, 2, 11 and 12 begin during diastole and end during systole; 3 and 4 are purely systolic; 5 and 6 begin during systole and end during diastole; 7, 8, 9 and 10 are purely diastolic.

Summary and conclusions.

In previous communications from this laboratory it has been shown, by means of hot-wire measurements of the inflow into the perfused left coronary artery, that the heart offers the greatest resistance to the blood flow during the period of systole. These experiments were repeated and no difference was found in this respect between the heart-lung preparation and the whole animal. In further experiments it was shown that, with small differences, the same holds true for a heart in which the coronary artery gets its blood supply from the aorta. The results of the experiments described in this communication lend further support to our former observations by showing that:

(1) Repeated obstructions of the coronary blood flow which occur in diastole conspicuously reduce the blood supply through the artery; on

the other hand, equal obstructions which occur during systole have almost no effect upon the blood flow. This result demonstrates that the blood supply to the heart during systole is negligible.

(2) Measurements of the blood-pressure between the place of clamping and the heart muscle show that during systolic clamping there is no change or sometimes even a rise of pressure in the coronary artery, while during diastolic clamping there is a precipitate fall of pressure. This result demonstrates that the heart muscle opposes the greatest resistance to the coronary blood supply during systole and the smallest resistance during diastole.

(3) These results were obtained in the heart-lung preparation and in the whole animal, in artificially perfused coronary arteries and in arteries which remained in connection with the aorta.

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AFFERENT IMPULSES IN THE VAGUS AND THEIR EFFECT ON RESPIRATION.

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In an early paper on sensory nerve impulses [Adrian, 1926] the writer gave a brief account of the discharge in the vagus during respiration. More detailed studies have been made by Keller and Loeser [1929] and lately by Partridge [1933], but neither of them gives records of the discharge in the individual nerve fibres. Without these it is difficult to know whether all the vagal endings are alike, and difficult to compare them with other sense organs. The present work deals with these points and with another question on which there has been some apparent disagreement, that of the existence of sensory endings responding to deflation of the lungs. The results confirm Keller and Loeser in demonstrating such endings, though the deflation needed to excite them can seldom occur in a normal animal. A final section deals with the effects of the vagal discharges on the respiratory centre.

Method.

Most of the experiments on the afferent discharge were made on cats, decerebrated or decapitated under chloroform and left until the effect of the anæsthetic had worn off. Some were made on rabbits anæsthetized with urethane, dial or chloralose, and these anæsthetics were also used in cats when the reflexes were investigated. In all but a few experiments a tracheal cannula was inserted and the movements of the lung were signalled by a rubber tambour leading from a side tube, the main branch being left open or connected temporarily to a 20-litre bottle. A small bottle (3 litres) was used to produce dyspnœa and various gases could be breathed from a Douglas bag. In many experiments artificial respiration was carried out by a pump with a rotary valve producing a known inflation at each stroke (Palmer's "Ideal" model). To study the effect of

increased or diminished external pressure on the thorax the animal was placed in an air-tight tank as in the experiments of Hammouda and Wilson [1932].

Except in the experiments on vagal reflexes the nerve was cut high up in the neck and dissected out for about 4 cm. To obtain the discharge in individual fibres it was subdivided with fine needles after removal of the sheath. Small bundles of nerve fibres are split off the main trunk, and in the cat it is nearly always possible, by further subdivision, to reach a stage at which the impulses form a single series. In the moist atmosphere of the incubator in which the animal is placed the nerve remains in good condition for several hours. Persistent discharges from the cut end seldom occur and can be abolished by further stripping of the sheath. The impulses were recorded in the usual way with a condenser-coupled amplifier, a Matthews oscillograph and a loud speaker. Silver silver-chloride Ringer electrodes were used with worsted leads to the nerve.

Results.

A detailed study of the discharge in single fibres was made only in the cat, for the slower rate of breathing makes it easier to analyse the sequence of events than in the rabbit. The general behaviour of the sensory endings is the same in the rabbit, though the existence of a separate cardiac depressor nerve is an important anatomical difference. Since the cat's vagus contains other afferent fibres besides those concerned in respiration it will be convenient to describe first the various types of afferent discharge which are not purely respiratory.

Discharges without the respiratory rhythm.

The greatest activity in the vagus always occurs at inspiration, and the majority of the fibres which yield measurable action potentials are evidently connected with sense organs which are excited by the expansion of the lungs. But besides these there are groups of impulses occurring at each heart beat, irrespective of the state of the lungs, and there is sometimes a persistent discharge in a few fibres with no sign either of cardiac or respiratory rhythm.

Persistent discharges. The records in Fig. 1 C and D show one continuous series of impulses uninfluenced by the expansion of the lungs together with a respiratory discharge of the usual type in several fibres. The former came from an ending near the trachea or lung root: it was increased in frequency by pulling the trachea slightly upwards and

abolished by pressing it downwards (Fig. 1 D). Similar discharges have been found in three preparations, and in some others there have been persistent discharges arising from within the thorax but not affected by the position of the trachea or the expansion of the lungs. Some of them have changed in frequency when the animal was placed on its side, but with others the factors responsible for stimulation have not been made out. The majority of the endings which give persistent discharges are

Fig. 1. Records showing afferent discharges in the vagus not influenced by respiration, all from decerebrate cats. A. Preparation with many nerve fibres, some giving cardiac rhythm. The usual respiratory discharge occurs at the beginning and end of the record. B. Record at higher speed showing cardiac discharge in two or three fibres. C. Persistent discharge (frequency 80 per sec.) due to slight traction on trachea. A respiratory discharge occurs in other fibres in the middle of the record. D. Persistent discharge abolished by moving trachea downwards. Time marker (white lines) gives $\frac{1}{4}$ sec. intervals in these and in all records.

evidently receptors of the slowly adapting or postural type stimulated by mechanical deformation but placed in regions which are not affected by the movements of the heart or lungs.

Cardiac discharges. In some cats the cardiac fibres (*i.e.* those giving a pure cardiac rhythm) are grouped into a slender nerve which runs with the vagus but is easily separated from it. In other animals there is no separate sheath, but the cardiac fibres are grouped together, so that if the vagus is split repeatedly cardiac fibres will be present in one or two

of the bundles but absent in the rest. This arrangement is often present in the right vagus although the cardiac fibres are contained in a separate nerve sheath on the left side. Examples of the cardiac type of discharge are shown in Fig. 1 A and B. In Fig. 1 A some of the fibres come from the lung and signal inspiration whilst others give cardiac rhythm. The discharge in each fibre usually consists of three or more impulses at each heart beat: by shunting a fraction of the electrocardiogram into the electrode system it can be shown that the impulses coincide with each systole. The number of impulses at each heart beat is increased by raising the blood-pressure and reduced by lowering it, and in general the discharge agrees very closely with that in the rabbit's cardiac depressor and carotid sinus nerve [cf. Bronk and Stella, 1932].

Fig. 2. Record from single fibre of vagus (decerebrate cat) showing discharge with mixed respiratory and cardiac rhythm. A, normal breathing; B, dyspnœa. Signal line moves upwards on inspiration: in these and other records it has been drawn in where it is too faint for reproduction. The duration of each record is 3 secs.

Discharges with mixed respiratory and cardiac rhythm. Since the structures near the root of the lung will be deformed by the pulsation of the heart and great vessels as well as by the movements of respiration we might expect to find some endings which are stimulated by both events. These are often found and the type of discharge they give can be seen from the records in Fig. 2. The cardiac rhythm is less constant than the respiratory and may disappear when the position of the animal is changed. The fibres which give this double rhythm form only a small minority of those in the vagus and in many preparations they have not been detected.

Respiratory discharges: endings stimulated by inflation.

In natural breathing (cat or rabbit) the frequency of the impulses in the vagus rises to a maximum at inspiration and falls to a minimum as the lungs contract. This is true both for quiet breathing and for extreme

dyspnœa induced by CO_2. In the whole nerve there are usually a good many fibres in which the discharge never ceases entirely during the interval between inspirations, though in dyspnœa the number becomes less and less as the deflation of the lungs at expiration becomes more complete. Typical records in which the impulses form a single series are given in Fig. 3 and a record from the rabbit's vagus is included for comparison (Fig. 3 A). Curves of impulse frequency are given in Fig. 4. The frequency rises and falls along a smooth curve as the lungs expand and

Fig. 3. Normal respiratory discharge in single fibres of the vagus. A, from a rabbit under urethane; B, C and D, from decerebrate cats. White line signals respiration and moves upwards when lungs expand (tambour connected to air reservoir). Time marker gives ½ sec. intervals.

contract, and the records might well have come from the stretch receptors of a muscle extended and relaxed rhythmically. The likeness remains when the expansion of the lungs is controlled by inflating them artificially. In Fig. 5, for instance, it can be seen that the frequency depends on the degree of inflation and falls off very slowly when the inflation is maintained.

We are dealing therefore with an ending which is like the muscle spindle in having a very slow rate of adaptation to the stimulus. The stimulus is evidently the actual deformation (stretching) of the tissues in which the endings are placed. Air currents are not essential, for the

discharge continues when the lungs are held inflated. The pressure of the air in the alveoli is also immaterial (except in so far as it produces

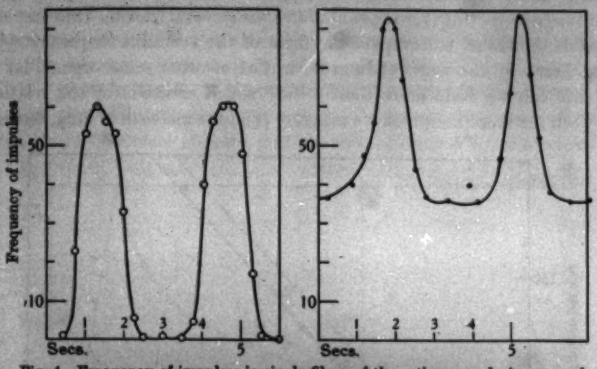

Fig. 4. Frequency of impulses in single fibres of the cat's vagus during normal breathing. Two preparations, both decerebrate.

Fig. 5. Spinal cat, single fibre preparation. Inflation of the lungs by pump. Movement of signal line directly proportional to inflation.

A.	Inflation = 65 c.c.	Maximum frequency	80	per sec.
B.	„ = 115 c.c.	„	120	„
C.	„ = 230 c.c.	„	250	„

deformation), for the discharge is the same for a given degree of expansion whether this is produced by blowing air into the trachea or by the natural movement of respiration.

The relation between the degree of inflation and the frequency of the discharge in single fibres is shown in the curves in Fig. 6. It is approximately linear over the range investigated, and in this it agrees with Partridge's findings for the rabbit's vagus (several fibres). It is scarcely possible, however, to compare the form of the stimulus-frequency relation shown by the vagal endings with that of other sense organs, for in no case can we form more than a very rough estimate of the relation between the stimulus which we measure (volume increase of lung, tension

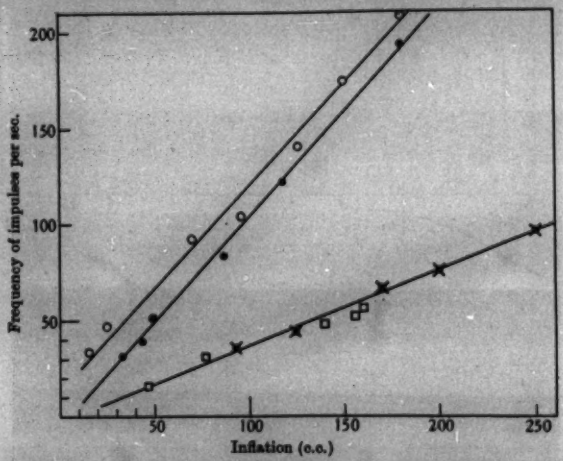

Fig. 6. Maximum discharge frequency in single fibres with varying inflation of lungs. Spinal cats. The two upper curves are from a small animal and show two series of measurements made within 10 min. The lower curve (also two series) is from a larger animal.

on muscle, etc.) and the deformation which it will produce in the sensory endings.

Adaptation. Keller and Loeser [1929] state that the discharge in the rabbit's vagus depends on the rate of inflation of the lungs as well as on the final extent, but Partridge [1933] finds no difference in the maximal frequency whether the inflation is slow or rapid. The discrepancy probably arises from differences in the range of stimuli employed, for in single fibres of the cat's vagus there is evidence of adaptation, but with stimuli of moderate intensity the effect is slight. When the degree of inflation

does not produce a frequency higher than about 50 per sec. there is little difference in the maximal frequency caused by an inflation developing in $\frac{1}{2}$ sec. or 3 sec., and after 10 sec. maintained inflation the frequency has seldom fallen by more than 5 p.c. But with rapid inflations, giving higher frequencies, there is an early decline from the maximum as there is in the discharge from a stretched muscle spindle. Curves illustrating this are given in Fig. 7. It is possible that the initial decline may be due in part to a redistribution of air in the lungs allowing the areas first expanded to contract slightly, but the rate of decline is not appreciably

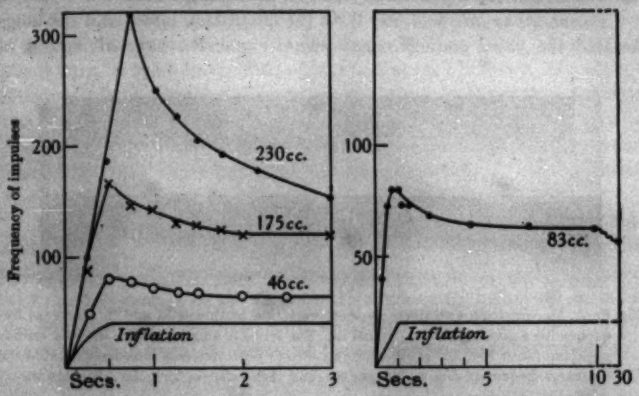

Fig. 7. Frequency curves from single fibres to show adaptation to stimulus. Spinal cat.

changed by binding the abdomen. After the initial decline the rate remains constant or falls slowly provided that the endings are in good condition. The fall is much more rapid when the circulation has failed (see later, p. 341).

Wedensky effect. In a few preparations an interesting result has followed from an extreme inflation of the lungs. The frequency rises to 300 per sec. or more and then falls suddenly, the discharge becoming irregular and finally ceasing altogether (Fig. 8 B). If some of the air is allowed to escape, the regular discharge reappears at a frequency in the neighbourhood of 50–100 per sec. This result is obviously an example of the Wedensky effect, closely resembling that found by Tsai [1931] when impulses from a frog's muscle spindle are recorded in a nerve fibre which has been led through a narcotizing chamber. In Tsai's experi-

ments a discharge of low frequency could pass the narcotic block, but if the frequency was raised by increasing the tension on the muscle conduction failed, either partially or completely, to return again when the tension on the muscle (and therefore the frequency of the incoming discharge) was lowered. In the present experiment it is conceivable that the failure occurred in the region of the nerve ending, but more likely that some impairment of conduction was produced in the nerve fibre by the dissection. Even if the fibre escaped all damage, the cut ends of other fibres might affect it by causing an electrotonic block or by raising the concentration of potassium ions etc. in its neighbourhood.

Variations in threshold, etc. With the circulation intact and the lungs aerated the vagal endings which signal expansion have all agreed in

Fig. 8. A. Record from two fibres to show variation in threshold and in discharge frequency for a given stimulus. Spinal cat. The series of small impulses shows a cardiac grouping (as in Fig. 2). B. Wedensky effect. With extreme inflation (250 c.c.) the discharge frequency rises to 300 per sec. and then becomes irregular. Spinal cat.

showing a very slow rate of adaptation. Judged by this criterion all of them are of the same type. There are, however, considerable differences in excitability, or rather in the amount of inflation needed to stimulate the different endings. This may be seen from Fig. 8 A which shows the discharge in two vagal fibres during a gradual inflation.

Whatever its cause, this variation in threshold will evidently increase the range of the signalling mechanism, but it may well depend mainly on the position of the endings, the actual deformation required to excite being the same for all of them. A change in the position of the animal will often produce a change in the response of a single ending to a given inflation. Otherwise the response remains constant over periods of an hour or more.

The effect of CO_2, *and of circulatory failure.* Bronk and Stella [1932] in their study of the stretch receptors in the carotid sinus have shown

that these endings are very little affected by changes in the acidity or gaseous content of the blood. Their function is to signal the expansion of the artery and nothing else. In the same way the stretch receptors in the lungs are extremely insensitive to environmental changes other than mechanical deformation. This was clearly shown in Head's work [1889] where it was found that inflation of the lungs with N_2 or H_2 produced the usual inhibition of inspiration. Keller and Loeser have found that in the rabbit the substitution of N_2 for air or the addition of 5–10 p.c. CO_2 causes no change in the vagal impulses, and Partridge agrees with them. In the cat (spinal curarized preparation) inflation with air containing 10–12½ p.c. CO_2 for several minutes seems to produce a very slight diminution in the discharge. This is shown by a fall of about 5 p.c. in the total number of impulses at each inflation. The change is scarcely large enough to be outside the range of error: it is certainly too small to be the basis of any considerable change in the depth of breathing. But as Keller and Loeser have pointed out, in the intact animal CO_2 may affect the vagal discharge indirectly by causing alterations in the calibre of the bronchioles.

Failure of the oxygen supply is another factor which has remarkably little effect on the vagal endings. The air may be shut off or nitrogen substituted for it until the heart has ceased to beat, without causing any decided change in the response to a measured inflation. In mammalian muscle Matthews [1931] has found that, if the effect of the anæsthetic has worn off, arrest of the circulation leads invariably to a spontaneous discharge of high frequency from all the sensory endings. With the vagal endings the only suggestion of this is a persistent discharge of low frequency (20 per sec.) which develops in some of the fibres when the lungs have been inflated rhythmically with nitrogen. The difference is easily explained, for Matthews found that the discharge from the muscle receptors was caused or greatly favoured by something produced in the muscle fibres and produced much more rapidly if they are made to contract. Since few of the vagal endings are likely to be exposed to metabolites from muscle there is no reason to expect great spontaneous activity in many of them.

The vagal endings cannot, of course, survive indefinitely after the failure of the oxygen supply and they become inactive within half an hour to an hour after the heart has stopped. The first sign of deterioration is a more rapid decline in the frequency of the discharge under a constant stimulus. A series of curves illustrating this are given in Fig. 9. They show the same change as Bronk's curves [1929] for the effect of nitrogen

on the response of a frog's muscle spindle, but it is surprising to find so little evidence of impairment 20 min. after the failure of the heart and the replacement of the air in the lungs by nitrogen.

Chloroform. In animals with the circulation intact very high percentages of chloroform vapour are needed to affect the vagal discharge; when it is affected it is usually abolished rapidly, and there is an immediate recovery as soon as the chloroform is withdrawn.

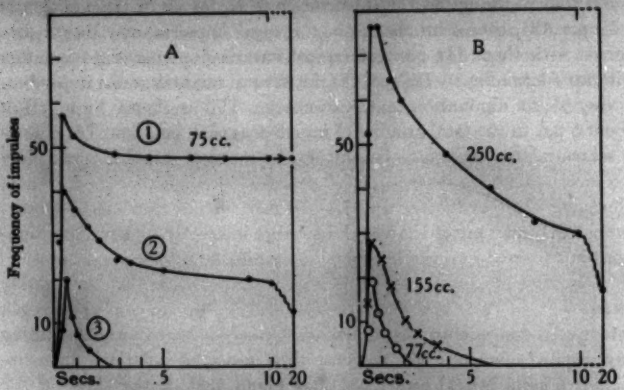

Fig. 9. Frequency curves from single fibre to show the effect of failure of circulation. Spinal cat. Lungs filled with nitrogen after death.

A. (1) Effect of inflation with 75 c.c. air with circulation intact.
(2) " " nitrogen 20 min. after heart failure.
(3) " " nitrogen 50 min. "
B. 50 min. after heart failure. Varying inflations with nitrogen.

Chloroform mixtures were breathed from a Woolf's bottle or blown into the lungs by a pump. The concentration was estimated roughly by weighing samples of the mixture collected in a 250 c.c. flask [Waller, 1904]. With decerebrate animals breathing a mixture of moderate strength the vagal discharge at each inspiration becomes less and less as the anæsthesia deepens, but this is due simply to the shallower breathing, for the response to a test inflation remains quite unaffected after the stage of complete flaccidity is reached and even after the stage at which respiration fails. With spinal animals under artificial respiration a mixture containing less than 2·5 p.c. of chloroform has no appreciable effect on the vagal discharge, though it abolishes all reflex activity. A mixture of 4 p.c. or more produces an immediate decline in the vagal discharge,

and within a minute or two it fails completely. It must be remembered, however, that during this time the lungs are still being inflated with undiminished force by the pump: the conditions are therefore quite unlike those in a patient breathing naturally. If the chloroform is turned off within a minute or so after complete failure there is an immediate recovery of the vagal discharge and within another minute it is back at its initial value. The rapid failure and recovery are illustrated in the records given in Fig. 10 which show a return almost to normal within

Fig. 10. Rapid failure with strong chloroform vapour (5 p.c.) and rapid recovery with air. Spinal cat. Lungs inflated by pump. A, with air; B, 70 sec. after chloroform has been turned on; C, recovery after 10 sec. inflation with air.

10 sec. Attempts to maintain a partial anæsthesia of the vagal endings were unsuccesaful. The lower concentrations of chloroform were too weak to affect them (though amply strong enough to affect the central nervous system) and the higher caused a rapid failure. This may be due to the rate at which the chloroform is absorbed by the blood stream, but it is difficult to produce a graded anæsthesia of a nerve trunk with chloroform vapour, and the action on the vagal endings may be equally abrupt.

The practical outcome of these experiments is to show that the strength of chloroform normally administered to a patient (*i.e.* below about 2·5 p.c.) is without effect on the endings in the lung which respond to mechanical stimulation. Very high percentages may affect them, but a few inflations with air should be enough to give complete recovery.

Endings stimulated by deflation of the lungs.

In the brief account given by the writer in 1926, it was stated that in normal breathing there is no sign of a renewed discharge of impulses at the moment when the lungs are most deflated, and that forcible deflation

of the lungs in a spinal cat does not give rise to a discharge. The forcible deflation referred to was that produced by compressing the thorax by hand, and this, in the cat, is not enough to stimulate the endings which respond to deflation. But Keller and Loeser showed that suction of air from the lungs undoubtedly gives rise to a discharge of nerve impulses. Their experiments were made on rabbits, and in the present work it has been found that the result is equally true of the cat: forcible deflation of this kind does produce a discharge.

In the cat when the thorax is opened the discharge can only be produced by deflating the lungs completely. It then varies rhythmically as each pulsation of the heart moves the collapsed lung. When the thorax is intact the discharge is usually continuous as long as the suction is maintained (Fig. 11), though in some preparations there has been only a short outburst at the beginning. From this it appears that some of the nerve endings are of the more rapidly adapting type, but the majority

Fig. 11. Afferent discharge produced in several fibres by suction of air from the lungs. Spinal cat.

are like those responding at inspiration. It is possible and indeed likely that some of the endings which react to suction are those which react normally to inflation, for both inflation and extreme deflation might stretch the tissues in which they lie. But there is no doubt that deflation calls a new set of endings into play as well. When only a few fibres are in action the impulses due to inflation and suction can often be distinguished by their size and form; also suction of air from the lungs has a specific effect on the respiratory centre. If the vagi are intact it causes an immediate contraction of the muscles which produce inspiration, whereas an inflation of the lungs has the reverse effect, since it inhibits inspiration. Thus the endings concerned in the response to extreme deflation must form part of a distinct nervous mechanism, though it is a mechanism which must be very rarely called into play.

The rabbit agrees with the cat in showing no expiratory discharge in normal breathing or in dyspnœa [Keller and Loeser, Partridge], but it differs from the cat in that it is usually possible to produce one by increasing the external air pressure on the thorax and abdomen so as to

assist the collapse of the lungs. The animal is placed in a closed chamber with the tracheal tube communicating with the air outside, the arrangement being a copy of that used by Hammouda and Wilson in their study of the vagal reflexes. Raising the air pressure in the chamber assists the collapse of the thorax at expiration and hinders the expansion at inspiration. The records in Fig. 12 were made from the vagus of a rabbit under these conditions, and it will be seen that with increased external pressure the usual discharge at inspiration is greatly reduced and in its place a definite expiratory discharge appears. In the cat this method has always failed to give an expiratory discharge, owing presumably to the greater strength of the body walls.

Whether the endings which are stimulated by collapse play much part in normal breathing is exceedingly doubtful. In the decerebrate or

Fig. 12. Afferent discharge at expiration produced by increased external pressure in the rabbit. Rabbit (under urethane) in air-tight chamber with trachea tube leading outside. A. Normal extra-thoracic pressure. Usual inspiratory discharge. B. Increased extra-thoracic pressure. Expiratory discharge and quickened breathing.

anæsthetized animal they play none at all except in conditions which are definitely abnormal. The failure to record a discharge at expiration has not been due to lack of activity in the expiratory muscles, for these have often contracted vigorously. It is possible that the forcible deflation occurring in extreme dyspnœa might stimulate the expiratory endings, but so far this has not been observed. On the other hand it is highly probable that these endings may come into play in various pathological conditions, *e.g.* when portions of the lungs are solidified or tied to the chest wall by adhesions.

It should be noted here that suction of air from the lungs (or breathing into a reservoir containing air at reduced pressure) will not necessarily have the same effects as increased pressure applied outside the thorax. Suction may well stretch some of the endings which are normally stretched at inspiration, but increased external pressure is unlikely to

do so. This difference may explain some of the divergent results which have been reported with the two procedures [cf. Hammouda and Wilson, 1932; Creed and Hertz, 1933].

The stretch receptors and the respiratory centre.

So much has been written about the action of the vagus in respiration that a brief discussion cannot hope to do justice to earlier work. It can only be excused by the fact that a more detailed knowledge of the afferent discharge gives a better foundation for analysing the vagal effect. We know that inflation of the lungs will produce a sustained discharge from the stretch receptors and that the deflation normally occurring will merely put an end to this discharge, though suction of air from the lungs will excite another set of endings. In normal conditions, therefore, the effect of the vagus must depend mainly, if not entirely, on the impulses from the stretch receptors. Persistent discharges unaffected by lung movements have been found in a few preparations and there is always the possibility of action currents too small to detect. But the dominant activity in the vagus is that due to the stretch receptors, and it is worth considering whether their discharges are enough to account for the whole of the vagal effect. In particular, can they account for the fact that more rapid breathing occurs when the vagi are intact than when they are cut? The messages from the stretch receptors have an inhibitory rather than an excitatory effect. Why should more rapid breathing be possible in their presence than in their absence?

The question was discussed by Hammouda and Wilson [1932]. Their evidence, based on the reactions to altered extra-thoracic pressure, led them to a picture of the sensory mechanism of the vagus which agrees in the main with that given here. But to explain the slowing of breathing on division of the vagi they suggest that the vagus has a double influence on the respiratory centre, an inhibitory influence varying in intensity with the lung volume, and besides this an influence which maintains the tone of the centre and augments the frequency of the discharge. The latter is considered to remain in general at a constant level of intensity unaffected by changes in the tension of the lung tissues.

Such an influence might be exerted by impulses in nerve fibres too small to give measurable action currents. It is quite possible, however, that the one sensory mechanism is enough: that the quickening of the rhythm is due to impulses from the stretch receptors and results from their inhibiting each period of inspiratory activity in the brain stem. An explanation on these lines was in fact given in the classical paper

published by Henry Head from Hering's laboratory in 1889, though it appeared then that the expiratory endings might play some part in the effect.

Later evidence has confirmed the main points in Head's theory. In the first place there is now no reason to doubt the power of automatic action by the respiratory centre. We are dealing with a group of neurones in the brain stem which tend to discharge periodically without the need for any periodic inflow of afferent impulses. Winterstein [1911] has shown that a recurrent discharge of motor impulses takes place in the phrenic nerve although the vagi have been cut and all movement has been paralysed by curare (and this has been confirmed in the experiments of Adrian, Bronk and Phillips [1932] as well as in the present work). During complete motor paralysis and a temporary pause in the artificial ventilation afferent impulses will still reach the central nervous sytem from pressure receptors, etc., but there will be no waxing and waning of the incoming stream to determine the period of the phrenic discharge. This is slower than that of normal breathing but no slower than that of breathing after division of the vagi. Thus the respiratory centre (or some part of the brain stem apparatus concerned in respiration) must be capable of beating with its own inherent rhythm. Its cells cannot be regarded as passive agents controlled entirely by afferent messages, for internal changes must be constantly taking place tending to produce a recurrent development of the active state.

Without the vagi the centre usually beats at a slow rate and only small variations of rhythm are possible. The messages from the stretch receptors change this spontaneous beat to the more rapid and flexible rhythm of normal breathing. They are inhibitory, but they can influence the rhythm in either direction because inhibition of the phase of activity will shorten this part of the cycle and tend to hasten its return, but inhibition during the phase of rebuilding will delay it. The dual influence discussed by Hammouda and Wilson depends, on this view, not on a dual afferent mechanism, but on the dual effect of inhibition on the respiratory centre. In Head's words the vagus inhibits inspiratory activity but at the same time raises the vitality of the centre by causing this activity to accumulate.

The maximum inhibitory effect occurs during the phase of activity and therefore the rhythm is usually quicker with intact than with cut vagi. The motor discharge will be curtailed, there will have been a smaller expenditure of active material and it is reasonable to suppose that less time will be taken in preparing for a fresh outburst. Thus a

series of brief inhibitions at each expansion of the lungs should give a cycle consisting of brief motor discharges repeated at short intervals; whereas the absence of all inhibition should give longer discharges spaced at greater intervals. This is the characteristic effect of division of the vagi, for the slowing is usually due to a prolongation of both the active and inactive phases of the cycle.

Fig. 13 illustrates the slowing of the rhythm due to the abolition of the vagal control and the quickening due to recurrent inhibition. The

Fig. 13. Discharge of motor impulses in the top root of the phrenic nerve cut distally. Decerebrate cat. All records at same speed. A. Both vagi blocked with novocaine. Slow rhythm (27 per min.) unaffected by sudden inflation of the lungs. Downward movement of signal marks inflation. B. Vagi intact. Normal breathing 58 per min. C. Vagi intact. Periodic inflation of lungs timed so as to inhibit each motor discharge. Downstroke of signal shows inflation. The rate increases to 90 per min. D. After 11 sec. the periodic inflation is stopped. The rate falls at once to 48 per min. Time marker (top record) gives $\frac{1}{4}$ sec. intervals.

records show the discharge of motor impulses in the top root of the phrenic in a decerebrate cat. When the vagi have been temporarily blocked with novocaine the motor discharges recur at 25 per min., each lasts for $1\frac{1}{2}$ sec. and the cycle is quite unaffected by inflation of the lungs. With intact vagi the inhibition normally occurring at each breath cuts short the motor discharge after it has lasted only $\frac{1}{4}$ sec. The rate of breathing is now 58 per min. By blowing air into the lungs at each inspiration the inhibitions are increased in intensity, the motor discharges are shortened still further and the rate rises to 90 per min., falling at once to 48 as soon as the inflations are discontinued. It will

be seen that both parts of the cycle are affected: the change in rate is due mainly to the shortening of the phase of activity, but there is some reduction in the interval between the end of one discharge and the beginning of the next. This is shown more clearly in the records in Fig. 14 which are made at higher speed.

This is little more than an example of the well-known fact that in an animal under artificial respiration the rhythm of the active movements of the diaphragm may come into phase with that of the pump. It shows that an increase in the inhibitory message at each inspiration can produce a quicker rhythm: it is therefore unnecessary to suppose that the vagus exercises its tonic effect on the centre through a distinct set of nerve fibres.

Fig. 14. Same experiment as Fig. 13. Records at higher speed (time marker gives $\frac{1}{4}$ sec.). Signal line drawn in. A. Normal discharge of motor impulses in phrenic root. B. Effect of periodic inflations. Both active and inactive phases are shortened.

The opposite effect will be produced if the inhibitory message acts throughout the period when the centre is recharging, for this will tend to delay the appearance of a fresh discharge. Thus the rhythm, in so far as it is determined by the stretch receptors, will be most rapid when the inhibitory effect rises sharply at each inspiration and falls to zero at expiration (Fig. 15 A) and slowest when the rise is less pronounced and there is some persistent inhibition throughout the cycle (Fig. 15 B). The frequency of the afferent discharge varies with the lung volume (p. 338). Therefore, if we can assume that the inhibitory effect does not much outlast the afferent discharge, the curves in Fig. 15 might relate to lung volumes instead of to inhibitions and the quickest rhythms should occur when the lung volume changes as in Fig. 15 A. The inhibition does no doubt outlast the afferent discharge, but records such as those in Fig. 13 show that it can rise and subside rapidly enough to affect only a limited part of the cycle. The conclusion given above is best illustrated by two

examples, the change in rhythm produced by altering the external pressure on the thorax and that occurring in CO_2 dyspnœa.

Increased pressure on the thorax. The effects of this have been studied in detail by Hammouda and Wilson, using the air-chamber method. They worked on dogs and found that an increase or reduction of pressure in the chamber caused an immediate quickening or slowing of the breathing. The changes only occur with intact vagi and they occur before the change in ventilation can have any effect.

A number of experiments have been made on cats by the same method, the afferent discharges in the vagi being sampled by recording the impulses in a few fibres slit from the side of the nerve. The earlier

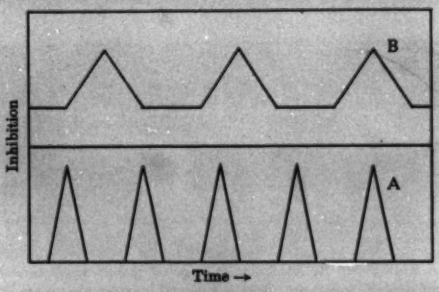

Fig. 15. B. Rise and fall of inhibition likely to give slow breathing.
A. Rise and fall of inhibition likely to give rapid breathing.

experiments failed to show the reaction to increased pressure and it was thought that its occurrence in other animals might depend on the stimulation of expiratory endings. But in later work, with chloralose as anæsthetic, the reaction was found in the cat, although there was never any sign of an afferent discharge produced by the deflation of the lungs. Fig. 16 gives typical curves showing the frequency of the vagal impulses (in several fibres) just before and just after the pressure in the chamber is raised. As might be expected, the whole frequency range is lowered when the thorax is reduced in volume, and in particular the discharge is much briefer and sinks to zero between each inspiration instead of remaining at a moderate level.

The quickening of the breathing is shown in the curves. It is evidently due to the reduced inhibition, but on the scheme outlined above it must be due not so much to the reduction in the peak of the inhibitory

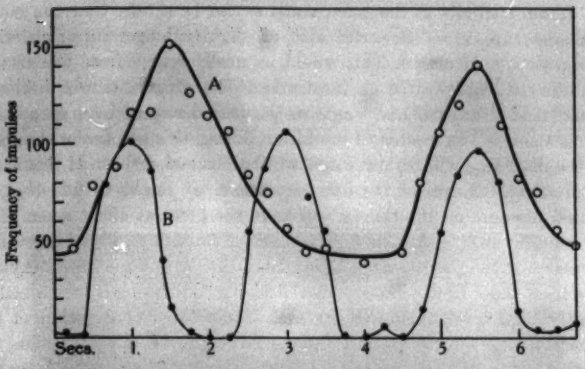

Fig. 16. Afferent vagal discharges in cat anæsthetized with chloralose. One vagus intact. Frequency curves (preparation of several fibres) showing the effect of increased extra-thoracic pressure on the discharge and on the rate of breathing. Curve *A*, normal extra-thoracic pressure. Rate 15 per min. Curve *B*, 5 mm. Hg positive pressure outside thorax. Rate 27 per min.

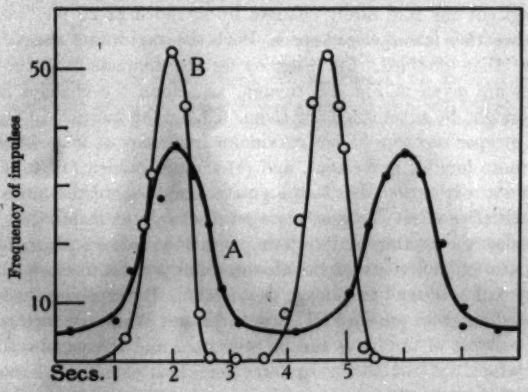

Fig. 17. Afferent vagal discharge in decerebrate cat showing the effect of dyspnœa due to rebreathing. One vagus intact. A. Normal, rate 15 per min. B. Dyspnœa, rate 22 per min. The impulses have been counted in two inspirations only.

effect as to its reduction in the later part of the cycle. By recording the action currents in the intercostals it can be shown that the motor discharges are more powerful and of slightly longer duration when the pressure is increased. This would naturally follow from the smaller inhibitory discharge at each inspiration. The greater motor discharge should tend if anything to lengthen the period required for rebuilding, but as there is less residue of inhibition during this phase the threshold for the discharge will be lower and will be reached earlier. If this is the factor which determines the increase in rate we ought to find that increased pressure on the thorax will have the greatest effect when there is a considerable background of persistent inhibition. This is certainly the case, for the preparations which have shown the quickened rhythm have been those in which the vagal discharge has normally remained at a fairly high level during expiration. Some tonic contraction of the diaphragm and intercostals (as often occurs with chloralose) favours the reaction, since it keeps the lungs slightly expanded; where such tone is absent there is often no increase in rate with increased intrathoracic pressure though there is a considerable slowing when the pressure is reduced. A reaction of this kind is usually found when dial or urethane are used as anæsthetic.

Change of rate in dyspnœa. The quickening of breathing in CO_2 dyspnœa can be explained on the same lines. In the decerebrate cat with vagi cut the rate rarely changes by as much as 25 p.c. when the animal breathes into a closed space. With the vagi intact the rate may be more than doubled. Curves showing the frequency of the vagal impulses are given in Fig. 17, though, as before, the changes in lung volume might be substituted for them. Under the increased CO_2 there is (*a*) a steeper rise to a higher maximum frequency at inspiration, due to the more forcible movement, and (*b*) a more sudden fall to a lower minimum at expiration, due to the greater emptying of the lungs. If it had much after effect the greater vagal discharge at inspiration might tend to slow the rhythm by interfering with the recovery phase, but the cutting short of activity and the absence of any vagal discharge during recovery will both tend to quicken the rhythm. By exposing the thorax to reduced external pressure (Hammouda and Wilson's method) the greater collapse of the lungs can be prevented and the vagal discharge at expiration restored to its former value. This causes an immediate slowing of the rate, though as asphyxia supervenes the effect is less and less marked, owing, presumably, to the rapid building up of active material in the centre.

The stretch receptors and the expiratory muscles.

The emptying of the lungs may be an active as well as a passive process and the afferent discharge in the vagus may influence expiration as well as inspiration. It is a simple matter to record the action currents of the expiratory muscles with hypodermic needle electrodes, for these allow us to lead from the individual motor units in each muscle without dissection of the chest wall and without danger of interference from neighbouring muscles. By this method it can be seen that in cats and

Fig. 18. Records from the external intercostal muscles (inspiratory) and the triangularis sterni (expiratory) made with hypodermic needle electrodes in a cat anæsthetized with chloralose. Signal moves upwards when lungs expand. A. External intercostal. B. Triangularis sterni. C. Both needles in parallel to show time relations of inspiratory and expiratory contraction. The regular excursions are due to the electrocardiogram.

rabbits under chloralose (and lying on the back or side) the triangularis sterni and the internal intercostals are regularly in action during the expiratory phase of the cycle. In decerebrate preparations and in animals under dial and urethane they are occasionally in action, in deep chloroform anæsthesia never. When they are not in action during quiet breathing they are not usually brought into action by moderate degree of dyspnœa.

Records from a cat under chloralose are given in Fig. 18. Two hypodermic electrodes were used, one in the external intercostal muscle and one with its point a few mm. deeper so that it led from the triangularis sterni. In record C both needles were connected in parallel with the

amplifier so as to show the time relations of the two discharges more clearly. The respiratory signal records the pressure change in a 20-litre bottle connected to the trachea tube. In these records the expiratory discharge fills up most of the gap between inspirations. If chloroform is given in addition to the chloralose the discharge becomes progressively shorter and is limited to the period when the deflation is at a maximum. Eventually it disappears, and in very deep anæsthesia the contraction of the external intercostals also ceases leaving only the diaphragm in action.

The periodic contraction of the expiratory muscles, when it occurs with the vagi intact, continues like that of the inspiratory muscles after the vagi are divided. The expiratory activity preserves its phase relationship to the inspiratory and both are unaffected by the state of expansion

Fig. 19. Same preparation as Fig. 18 after section of both vagi. A. External intercostal. B. Triangularis sterni. Inflation or deflation of the lungs has now no effect on either discharge.

of the chest (Fig. 19). It is probable, therefore, that the expiratory discharge is as much a product of the automatic beating of the respiratory centre as the inspiratory. The nerve cells responsible for it are so linked to the nerve cells responsible for inspiration that the two groups cannot come into action simultaneously and normally the expiratory activity occurs as an after effect of the inspiratory. But it can also be made to occur independently by an afferent discharge from the stretch receptors. There is some variation in this respect between one preparation and another. In some an inflation of the lungs causes an immediate expiratory contraction even though it is timed so that the normal inspiratory contraction is completely suppressed. In others the expiratory contraction develops more slowly and an inflation of the lungs is a less potent stimulus than a period of inspiratory activity.

In his account of respiration in animals after various operations on the brain stem Lumsden [1923] states that electrical stimulation of the

vagus inhibits expiratory contractions. This may well be due to the effect of the nerve fibres coming from the endings which are excited by suction of air from the lungs. These cause an immediate reflex inspiration and an inhibition of the expiratory contraction, and on electrical stimulation of the vagus their effect may outweigh that of the fibres from the stretch receptors. The latter certainly favour expiratory as they inhibit inspiratory activity.

As far as the vagal control of breathing is concerned, the action of the expiratory muscles can be regarded merely as an additional factor tending to reverse the effect of inflation. In so far as it secures a smaller lung volume at expiration the contraction of these muscles will help to establish the type of vagal discharge which promotes rapid breathing. But in animals under chloralose there is often some tonic contraction of the diaphragm to oppose deflation. Thus the breathing may be no faster than it is in animals with no expiratory activity but more complete muscular relaxation.

Discussion.

As usual it is easier to reach definite conclusions about the sensory mechanism than about its action on the central nervous system. The main group of sense organs in the lung behaves no differently from the muscle spindles or stretch receptors in other parts of the body. They give the usual serial discharge of impulses when they are stretched, they become very slowly adapted to the stimulus, they are relatively unaffected by anæsthetics, lack of oxygen, etc. Their function is to signal the volume of the lungs at each moment and they continue to do this for half an hour or more after the heart has stopped beating. Since the volume of the lung changes relatively slowly, the endings must be of the slowly adapting or postural type. The only evidence of rapidly adapting endings supplied by the vagus is the occurrence of an initial brief discharge in some preparations when the lungs are collapsed by suction, but these have not yet been analysed in records made from a few nerve fibres.

The stretch receptors in the lung resemble those in muscle in their effect on the central nervous system. Both influence it to cut short the movement which has stimulated them, and by so doing prevent the inconvenience or damage which might come from unrestrained motor activity. But the lungs must be adequately ventilated, and if the range of movement is restricted the movements must succeed one another at shorter intervals. Without the vagal mechanism the respiratory centre (in the conditions of these experiments) slowly charges and discharges

itself with little variation of rhythm. The impulses from the stretch receptors quicken the rhythm by cutting short the discharge and so hastening the recharging process. They are inhibitory, but they seem to act mainly by raising the discharge threshold, not by preventing the accumulation of active material.

This view need not be further elaborated since it was stated with more detailed evidence in Head's paper. It postulates a type of inhibition which seems to agree in general with the results of Eccles and Sherrington on the limb reflexes. It is natural to compare the inhibition of the respiratory beat with that of the heart beat, and at first sight the results seem to differ radically, for the respiratory beat is quickened and the heart beat slowed. But the former is quickened by inhibition confined more or less to each period of activity; it is slowed if the inhibitory discharge fills up the whole cycle. When the heart is slowed by electrical stimulation of the vagus it is exposed to a steady inhibition comparable to that produced on the respiratory centre by holding the lungs inflated. The slow subsidence of the effect from single stimuli [Brown, Eccles and Hoff, 1932] makes it unlikely that the inhibition of the heart could be made to fluctuate rapidly enough in relation to the cycle to establish a fair parallel between the two cases.

Throughout the preceding discussion the term respiratory centre has been used as a name for that part of the central nervous system in which the rhythmic activity takes origin. Where this occurs is a matter for studies such as those of Lumsden [1923] and does not concern us here. Slow potential changes in phase with respiration can be detected in the brain stem of the rabbit and seem to resemble those found in the isolated brain stem of the goldfish by Adrian and Buytendijk [1930]. In the mammal, however, the brain stem cannot be isolated and it is a much more difficult matter to analyse the waves and to make sure that they are not artefacts due to movement of fluid, etc.

Summary.

The action of the vagus in respiration has been studied in the cat by recording the impulses in single afferent fibres.

1. Some of the discharges come from end organs which are not affected by lung movement, *e.g.* from end organs in relation to the heart and to the trachea.

2. Of the end organs which respond to lung movement and give measurable action currents, the only kind in action normally is that stimulated by expansion of the lung.

3. These end organs behave like the muscle spindles and stretch receptors in other parts of the body. They become very slowly adapted to the stimulus. In normal breathing the maximum frequency of the discharge in single fibres varies from 50 to 100 per sec., though rates as high as 300 per sec. are produced by extreme inflation. With moderate inflations the frequency varies directly with the lung volume.

4. Carbon dioxide in amounts higher than 10 p.c. produces a very slight diminution in the discharge to a test inflation, but the effect is not outside the range of error. Ventilation with nitrogen and failure of the circulation have no immediate effect. After 10–20 min. the discharge to a constant stimulus declines more rapidly, but there may still be some response an hour after the heart has stopped beating.

5. Chloroform vapour in amounts of 2·5 p.c. or less has no obvious effect on the response of the end organs. Higher proportions produce a rapid failure, but there is a rapid recovery as soon as the lungs are inflated with air.

6. Suction of air from the lungs stimulates a fresh set of end organs. These may come into action in pathological conditions, but they are not stimulated by the normal movement of the lung and there is no evidence of their activity in moderate dyspnœa. In the rabbit they are stimulated at expiration when the extra-thoracic pressure is increased.

7. The effect of the vagus on respiration must be due mainly to the impulses from the stretch receptors in the lung. These have an inhibitory effect on the respiratory centre, but the inhibition occurs mainly during the phase of activity. Periodic inhibitions may therefore quicken the rate of breathing, as Head suggested, by cutting short the motor discharge and so hastening recovery.

8. The expiratory muscles (triangularis sterni and internal intercostals) contract regularly in cats and rabbits anæsthetized with chloralose. They are sometimes in action in decerebrate cats but never in cats under deep chloroform anæsthesia. In chloralosed animals the expiratory muscles remain in action after section of both vagi.

9. The afferent control of the respiratory movements is discussed.

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THE RENAL ELIMINATION OF INJECTED UREA AND CREATININE.

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CONTENTS.

	PAGE
Introduction	360
Theoretical basis and methods	362
General details	363
Long sample experiment	364
Basic principles	364
Technique	365
Short sample experiment	366
Basic principles	366
Technique	367
Experimental results	368
Creatinine	368
Long sample experiments	368
The renal extraction ratios	368
The amounts of creatinine in the kidneys	369
The blood flow through the kidneys	370
Short sample experiments	371
The renal extraction ratios	371
The amounts of creatinine in the kidneys	372
Additional experimental results	372
Distribution of creatinine in the blood	372
Plasma volumes	373
Urea	375
Long sample experiments	375
The renal extraction ratios	375
The amounts of urea in the kidneys	376
The blood flow through the kidneys	376
Short sample experiments	377
The renal extraction ratios	377
The blood flow through the kidneys	378

PAGE

Relationships of the renal extraction ratios 379
Creatinine 379
Long sample experiments 379
Short sample experiments 380
Urea 384
Long sample experiments 384
Short sample experiments 384
Combined urea and creatinine experiments 388
Plasma renal extraction ratios 391
Creatinine 391
Urea 393
Interpretation of the renal extraction ratios 394
Ratios later than 50 minutes after the injection 395
Creatinine 395
Urea 397
Ratios during the first minute after the injection 399
Ratios at 2 to 20 minutes after the injection 400
General 402
Appendix 403
Consideration of experimental technique 403
Long sample experiment 403
Endogenous creatinine and urea 403
Injected creatinine and urea 405
Short sample experiment 406
Renal vein blood sample 406
Heart blood sample 408
The effect of urea on peritoneal absorption 411
Chemical methods 412
Creatinine 412
Blood and urine 412
Kidneys 412
Urea 413
Blood 413
Kidneys 413
Summary 414
References 415

INTRODUCTION.

THE subject of the present paper is the experimental determination of the renal extraction ratios for injected creatinine and urea, *i.e.* the proportion of these substances taken up by the kidneys from the blood passing through them. Urea or creatinine was given intravenously to

rabbits, and at various times afterwards the percentage of the substance in the renal vein blood was compared with the percentage in the arterial blood.

The elimination of creatinine has not been studied in this way by previous workers: that of urea has been studied by Picard [1856], Addis and Shevky [1917], Rhoads, van Slyke, Hiller and Alving [1931], and Dunn, Kay and Sheehan [1931]. Of these, the work of Picard is of only historic interest. That of Addis and Shevky will be considered in detail later. Rhoads, van Slyke, Hiller and Alving utilized an excellent technique, but only their main conclusions are as yet available. They state that the proportion of urea removed from the blood during its passage through the kidney remains constant whatever the height of the blood urea, apart from a few occasions when there is a complete cessation of removal of urea from the renal blood, and the renal vein blood urea rises as high as, and sometimes higher than, the arterial blood urea. It should be noted that these workers used dogs from which one kidney had usually been removed; their findings are in certain respects different from the results obtained with rabbits with two functioning kidneys reported in this paper. The work of Dunn, Kay and Sheehan dealt with the elimination of urea in rabbits with no previous administration of urea.

The excretion of urea in the urine has been studied by many workers: that of creatinine has been the subject of much recent enquiry [Major, 1923; Heesch and Tscherning, 1926; Rehberg, 1926; Cope, 1931; Shannon, Jolliffe and Smith, 1932, etc.].

TABLE I. Urinary excretion of urea and creatinine. Mean figures for five rabbits observed 16 days each.

		Daily urine			
Rabbit	Wt. kg.	Volume c.c.	Urea mg.	Creatinine mg.	Ratio $\frac{\text{Urea}}{\text{Creatinine}}$
A 1	2·05	71	1870	106	18
A 2	2·27	87	1410	75	19
A 3	2·00	120	1810	84	22
A 4	2·72	118	2320	139	17
A 5	2·04	84	1230	69	18
Mean	2·20	96	1730	95	18

The normal excretion of creatinine by the rabbit has not been previously investigated very thoroughly, and it was therefore considered useful to observe five normal animals for a period of 16 days each; the figures are summarized in Table I.

The daily amounts of urine and of the urea and creatinine were rather variable, but the ratio of urea to creatinine in the urine was much more constant both from animal to animal and from day to day, and averaged 18. The average daily excretion of creatinine was 43 mg. per kg. of body weight. It is not possible to make any accurate calculations from these data as to the relative plasma clearances of creatinine and urea at normal levels in the rabbit, as the usual "blood creatinine" in this animal is only about 1·3 mg. per 100 c.c., and possibly nearly half of this amount may be "chromogenic substance" [see Cope, 1931].

Attempts were made to compare the creatinine contents of arterial blood and renal vein blood in the normal rabbit, but these were unsuccessful as the creatinine contents are so low that accurate colorimetry is impossible. Thus figures cannot be given for comparison with the figures published previously for the renal elimination of urea at normal levels.

The present work deals with the renal elimination of urea and creatinine when these substances have been raised to abnormally high levels in the blood. The investigation thus gives information only about the activities of the kidney under the specific conditions of overload, and deductions from the results as to normal activities of the kidney may be made only with great caution. Nevertheless, these abnormal amounts of normal substances are presumably thrown on the normal renal mechanism, and the experiments may thus be considered to relate to the activity of this normal mechanism under strain. Alternatively, the abnormal amounts of the substances are dealt with by some accessory renal mechanism, which cannot be regarded as utterly abnormal, and whose scope of activity is a problem of importance in renal physiology.

THEORETICAL BASIS AND METHODS.

To find the renal extraction ratios two types of operative procedure were employed: the *long sample* and *short sample* experiments. These experiments consisted of injecting the substances into an ear vein, collecting blood from the left renal vein, and making comparisons as follows:

(1) *Long sample experiment:*

Between the total amounts of the substances

(*a*) carried by the blood to the kidney, and

(*b*) retained by the kidney,

during the whole period of the experiment.

(2) *Short sample experiment:*
Between the percentages of the substances in corresponding samples of
(*a*) renal artery blood, and
(*b*) renal vein blood,
at a specific time after the injection.

The general principles of these experiments have been described previously [Sheehan, 1931]. Certain modifications were necessary to suit the conditions of the present study; these are considered below. It will be seen from the basic principles of the experiments that the long sample experiment requires the injection into the blood stream of the substance to be examined, even if this substance is normally present in the blood. The short sample experiment, on the other hand, can be used to find the renal extraction ratio of any blood constituent either at its normal level or after raising the level artificially by an intravenous injection. For the sake of clearness, creatinine alone is referred to in the following account, but it is to be understood that the theoretical discussion applies also to urea or any other substance which might be used.

General details.

The animals used were rabbits weighing usually between 2 and 2·5 kg. They were anæsthetized by an intravenous injection of 10 c.c. per kg. of 20 p.c. solution of urethane in physiological saline given at a rate of 2 c.c. per min.; the operation was never begun earlier than 5 min. after the end of the injection of the urethane. Occasionally if an animal was left for over half an hour after giving the urethane, a small second injection of anæsthetic was required.

Careful study was made of the possible effect of variation in the time between giving the urethane and beginning the operation, and this point was further investigated experimentally after the effect of the other variable factors had been established. These controls showed that the variations in the method of giving the anæsthetic did not affect the experimental results at all.

Four experiments have been discarded as the animals showed gross chronic nephritis; in two of them the renal function was decidedly subnormal.

The renal extraction ratio was always calculated to the nearest whole number; this is about the limit of significance of any results.

Long sample experiment.

Basic principles.

If creatinine be injected into the blood stream *via* an ear vein, some of it will go in the blood along the renal artery to the kidney during, say, the next 60 sec. Of this, part will be taken up by the kidney parenchyma, part will pass on through the capillaries and leave the kidney by the blood in the renal vein. The amount leaving the kidney can be found by collecting all the blood leaving by the renal vein from the beginning of the injection into the ear vein to the end of the experiment, and determining the total amount of creatinine in this blood. The total amount taken up by the kidney parenchyma can be found by removing the kidney at the moment the collection of the renal vein sample is ended, performing the appropriate determinations on the kidney and applying a correction for the amount of creatinine in the kidney before the experiment. The sum of these two amounts is the amount of creatinine which came to the kidney *via* the renal artery during the experiment.

The renal extraction ratio is the ratio between the amount taken up by the kidney and the amount reaching the kidney during the experiment; expressed as a percentage it is

$$100 \times \frac{\text{Amount taken up by the kidney}}{\text{Amount taken up by the kidney} + \text{amount leaving kidney in renal vein blood}}.$$

The method of determining the correction for the creatinine and urea in the kidney before the experiment is discussed in the appendix. The allowance amounted to 0·4 mg. of creatinine and 8·8 mg. urea per kidney. Its effect is to make the renal extraction ratios a little lower than they would have been without correction; the greatest reduction in the case of creatinine is 3 p.c., and in the case of urea 7 p.c.

The long sample experiment is limited in its application to the first minute after the injection of the creatinine as the amount of blood removed is too large for the collection to be continued longer than a minute. On the other hand it is easier and more reliable than a short sample experiment performed during the first minute, for this latter experiment then requires the introduction into the calculations of a correction, which is large in comparison with the small differences between the creatinine contents of the heart and renal vein blood, and the possible error is thus too big for reliable conclusions to be drawn. Nevertheless, under absolutely standard conditions and when the substances to be examined are suitable, the two types of experiment give the same results, as has been shown in previous work on dyes.

Technique.

The technique of the operation is similar to that described by Sheehan [1931], but the renal vein blood and heart blood were collected by the improved method described by Dunn, Kay and Sheehan [1931]. In a number of cases the heart blood was obtained by opening the thorax and needling the left ventricle directly; this is a very efficient and rapid method of collecting heart blood, and the urea or creatinine content of the blood does not appear to be affected during the few seconds required for the collection. The syringes for collecting the blood contained 0·05 c.c. of 20 p.c. potassium oxalate solution; a correction was made in all figures for this volume. The amount of blood collected from the renal vein varied from 8·6 to 23·0 c.c.; the time of collection was usually 1 min. from the beginning of the injection of the creatinine into the ear vein.

In all the creatinine experiments the initial injection of creatinine was given in the standard time of 10 sec. A 10 p.c. solution in physiological saline was used. A 10 p.c. urea solution was used in all urea experiments except the long sample ones, in which a serious difficulty was found. The rapid intravenous injection of strong urea solutions produces a very marked renal vaso-constriction for about half a minute; the slow injection of a large volume of weaker solution produces much less effect. On the other hand, as will be explained in the appendix, it is important in the long sample experiments that the duration of the injection shall not be too long and that the volume of fluid injected shall not be excessively large. The technique which best satisfies the requirements of the experiment without producing severe interference with the renal blood flow was found to be the injection of 25 p.c. urea solution during a period of 30 sec. Nevertheless, the renal circulation rates have varied greatly in the course of each experiment; the rate at the time of the peak of the urea content in the blood cannot therefore be established.

Chemical analyses were performed to determine:

(*a*) the total amount of creatinine in the whole volume of blood collected from the left renal vein;

(*b*) the total amount of creatinine in the left kidney; and for purposes of control,

(*c*) the total amount of creatinine in the right kidney;

(*d*) the percentage of creatinine in the heart blood;

(*e*) the partition of the blood creatinine between the plasma and corpuscles.

The details of one actual long sample experiment are given below.

Typical protocol of long sample experiment.

R. 574. Female. Wt. 2·29 kg. 18. xii. 31.

Pre-operative.

0–12 min. 20 c.c. urethane 20 p.c. solution I.V.
50–52 „ 4 c.c. „ „ „
56 „ Long sample experiment.

Operation.

0 sec. Laparotomy commenced.
8–18 „ 5 c.c. creatinine 10 p.c. solution I.V.
20–22 „ 1 c.c. isotonic saline I.V. to wash through vein.
8–78 „ Collection of blood from left renal vein (23·0 c.c. in 70 sec.).

c.c. of blood collected	2	4	6	8	10	12	14	16	18	20	22
Time in sec.	11	16	21	26	32	39	46	54	61	68	75

78 sec. Left kidney removed.
79 „ Right kidney removed.
84–86 „ Collection of blood from heart.

Estimations.

Left kidney 9·3 g. 101 mg. creatinine per 100 g.
9·4 mg. „ total.
Right kidney 9·9 g. 104 mg. „ per 100 g.
10·3 mg. „ total.

[From each of these totals 0·4 mg. must be subtracted to allow for pre-existent creatinine in the kidney.]

Heart blood 3·2 c.c. 100 mg. creatinine per 100 c.c.
Renal vein blood 23·0 c.c. 122 mg. „ per 100 c.c.
28·1 mg. „ total.
Renal vein blood plasma 150 mg. „ per 100 c.c.

Renal vein blood has 66 p.c. of plasma.

Calculations.

$$\text{Renal circulation rate} = \frac{23{\cdot}0 \times 60}{9{\cdot}3 \times 70} = 2{\cdot}1 \text{ c.c. per g. per min.}$$

$$\text{Whole blood renal extraction ratio} = \frac{100\,(9{\cdot}4 - 0{\cdot}4)}{28{\cdot}1 + (9{\cdot}4 - 0{\cdot}4)} = 24 \text{ p.c.}$$

Renal vein blood plasma: total volume 15·2 c.c.
total creatinine content 22·8 mg.

$$\text{Plasma renal extraction ratio} = \frac{100\,(9{\cdot}4 - 0{\cdot}4)}{22{\cdot}8 + (9{\cdot}4 - 0{\cdot}4)} = 28 \text{ p.c.}$$

Short sample experiment.

Basic principles.

After creatinine is injected into an ear vein it circulates in the blood stream for some considerable time. Blood containing a high content of creatinine is thus continuously passing through the kidney and losing a certain proportion (the renal extraction ratio) of its creatinine. This proportion could in theory be found by collecting firstly a sample of blood from the renal artery, and secondly a sample of blood from the renal vein about 3 sec. later, during which interval the arterial blood would have had time to pass through the vascular bed of the kidney to the

vein. In practice, as has been shown in a previous paper [Sheehan, 1931], such an experiment is not possible in the rabbit. Actually it is necessary to collect the renal vein blood sample first, and a sample of arterial blood from the heart as soon as possible afterwards. If the injection is given several minutes before the operation, this heart blood sample has practically the same creatinine content as the heart blood just before the collection of the renal vein blood; if necessary a correction can be made for the small reduction which occurs during the time. The method of ascertaining the requisite correction is discussed in the appendix.

The calculation of the renal extraction ratio is a matter of elementary proportion.

$$\text{Renal extraction ratio, p.c.} = 100 \times \frac{\text{difference between creatinine content of heart blood and renal vein blood}}{\text{creatinine content of heart blood}}.$$

Technique.

The creatinine, in 10 p.c. solution, was injected from 2 to 160 min. before the operation; in this point the experiments differed from those previously reported with dyes, where the interval was only a few seconds. The operative technique is described by Dunn, Kay and Sheehan [1931].

The urea was also given in 10 p.c. solution in isotonic saline. The dose was usually 1000 mg. per kg., but was varied, in some cases, from 350 to 1820 mg. per kg. An attempt was also made to study the renal extraction ratio at very high blood urea levels, but the large amounts of urea which it was necessary to inject proved to be too toxic to the rabbits. In the whole group of short sample experiments the operation was performed at various times from 3 to 213 min. after the injection of urea. The blood ureas lay between 47 and 296 mg. per 100 c.c. Measurements of the urea in the kidneys were not made in any of these experiments.

Chemical analyses were performed to find:

(*a*) the percentage of creatinine in the heart blood;

(*b*) the percentage of creatinine in the renal vein blood; and, for purposes of control,

(*c*) the total amount of creatinine in the kidneys;

(*d*) the partition of the blood creatinine between the plasma and corpuscles.

The details of one actual short sample experiment are given below.

Typical protocol of short sample experiment.

R. 563. Female. Wt. 2·15 kg. 17. xii. 31.

Pre-operative.

0–10 min. 20 c.c. urethane 20 p.c. solution I.V.
63 „ 10 c.c. creatinine 10 p.c. solution I.V.
77 „ Short sample experiment (14 min. after creatinine).

Operation.

0 sec. Laparotomy commenced.
11–18 „ Collection of blood from left renal vein (2·5 c.c. in 7 sec.).

c.c. of blood collected	1	2
Time in sec.	14	16

18 sec. Left kidney removed.
20 „ Right kidney removed.
19–23 „ Collection of blood from heart.

Estimations.

Left kidney	6·5 gm.	162 mg. creatinine	per 100 g.	
		10·5 mg. „	total.	
Right kidney	6·5 gm.	162 mg.	„	per 100 g.
		10·5 mg.	„	total.
Heart blood	7·1 c.c.	121 mg.	„	per 100 c.c.
Renal vein blood	2·5 c.c.	113 mg.	„	„ 100 c.c.
Heart blood plasma		120 mg.	„	„ 100 c.c.

Heart blood has 61 p.c. of plasma.

Calculations.

$$\text{Renal circulation rate} = \frac{2{\cdot}5 \times 60}{6{\cdot}5 \times 7} = 3{\cdot}3 \text{ c.c. per g. per min.}$$

$$\text{Whole blood renal extraction ratio} = \frac{100\,(121-113)}{121} = 7 \text{ p.c.}$$

The plasma from 100 c.c. heart blood contains 61 p.c. of 120 mg., *i.e.* 73 mg. creatinine.

$$\text{Plasma renal extraction ratio} = \frac{100\,(121-113)}{73} = 11 \text{ p.c.}$$

EXPERIMENTAL RESULTS.

CREATININE.

Long sample experiments.

The renal extraction ratios.

Eight satisfactory experiments were performed, the more important details of which are given in Table II.

The renal extraction ratios during the first minute after the injection of the creatinine lie between 39 and 21 p.c., with a mean of 28 p.c. The significance of these renal extraction ratios will be considered later.

TABLE II. Creatinine long sample experiments.

Rabbit	Amount of creatinine injected mg.	Duration of collection of renal vein blood sec.	Amount of creatinine accumulated in left kidney (observed amount minus allowance of 0·4 mg. for pre-existent) mg.	Total amount of creatinine in renal vein blood mg.	Renal extraction ratio p.c.
B 1	500	38	13·1	20·6	39
B 2	500	40	6·4	13·8	32
B 3	500	25	9·7	26·2	27
B 4	200	60	3·3	8·8	27
B 5	200	60	4·2	11·9	26
B 6	500	60	7·2	21·9	25
B 7	500	70	9·0	28·1	24
B 8	500	66	8·1	30·1	21

The amounts of creatinine in the kidneys.

Some interest attaches to the rapidity with which the creatinine accumulates in the kidneys during the first minute after injection. Table III gives the percentage of injected creatinine found in the two kidneys together at various times after the injection.

TABLE III. Creatinine long sample experiments.

Rabbit	Mean time of removal of kidneys: sec. after beginning of injection	Amount of injected creatinine accumulated in kidneys (observed amount minus allowance of 0·8 mg. for pre-existent) mg.	Total amount injected mg.	Percentage of total amount injected accumulated in kidneys
B 1	40	25·4	500	5·08
B 2	40	13·4	500	2·68
B 3	26	18·8	500	3·76
B 4	61	6·2	200	3·10
B 5	61	8·9	200	4·45
B 6	63	15·0	500	3·00
B 7	70	18·9	500	3·78
B 8	69	16·0	500	3·20
Mean	54			3·63

The mean percentage of creatinine accumulated in the two kidneys together at about a minute after the injection was 3·63. The mean weight of the animals was 2·53 kg. and of their two kidneys together 17·0 g.; *i.e.* the kidneys were only 0·67 p.c. of the body weight but took up more than five times this percentage of the injected creatinine within a minute or less after the injection. This figure of 3·63 p.c. at 1 min. is in no way surprising. It is in satisfactory agreement with what is known of the rate at which other substances accumulate in the kidney. Thus, for example, during the first minute after injection, phenol red has a renal

extraction ratio about twice that of creatinine, and it does not disappear from the general blood stream quite so quickly [Sheehan, 1931]. It would therefore be expected that the amount of phenol red taken up by the kidneys within a minute after injection would be rather more than twice the amount of creatinine taken up in the same time, assuming normal renal circulation rates in both cases. Actually the mean amount of phenol red taken up is 8·4 p.c. of that injected [Sheehan, 1931], *i.e.* two and a third times the percentage of creatinine which accumulates in the kidneys in the same time.

The blood flow through the kidneys.

The renal circulation rates in these experiments were quite normal in range, as will be seen from Table IV. The actual rate of the blood flow from the renal vein was usually very steady; in only two animals did the flow slow down to any recognizable extent towards the end of the collection. In these two cases the collection was stopped at once.

Any such reduction of the renal circulation rate towards the end of the collection is probably due to generalized vasomotor disturbance. The blood flow to the left kidney does not appear to have been disturbed by the operative interference with its vein. This is shown by the close agreement between the amounts of creatinine which accumulated in the two kidneys from any animal. The relative amounts of creatinine in the two kidneys may be taken as a fairly good indication of the relative

TABLE IV. Creatinine long sample experiments.

Rabbit	Renal circulation rate c.c. per g. per min.	Total kidney creatinine left/right p.c.
B 1	1·7	106
B 2	1·7	92
B 3	3·4	106
B 4	2·0	112
B 5	1·9	90
B 6	2·0	93
B 7	2·1	91
B 8	2·4	103
Mean	2·15	99

blood flows through these kidneys, since the amount of creatinine in the blood is presumably the same for both sides. The amount of creatinine in the left kidney expressed as a percentage of that in the right kidney is shown in Table IV.

Short sample experiments.

The renal extraction ratios.

The results of the short sample experiments are collected in Table V, arranged according to the time elapsing between the injection of creatinine and the operation.

It will be seen that the renal extraction ratios lie between 6 and 23 p.c.

TABLE V. Creatinine short sample experiments.

Rabbit	Creatinine injected: mg. per kg.	Creatinine injected: min. before operation	Renal circulation rate c.c. per g. per min.	Creatinine mg. per 100 c.c. blood: Heart	Creatinine mg. per 100 c.c. blood: Renal vein	Renal extraction ratio p.c.
C 1	225	2	1·9	67	56	16
C 2	470	3	1·4	160	145	9
C 3	215	4	2·6	59	54	8
C 4	500	5	2·3	114	100	12
C 5	230	6	1·3	64	53	17
C 6	490	8	1·5	169	159	6
C 7	210	12	2·5	41	32	22
C 8	465	14	3·3	121	113	7
C 9	200	19	3·3	31	29	6
C 10	225	26	2·8	32	27	15
C 11	500	26	3·5	99	85	14
C 12	500	44	1·7	66	56	15
C 13	500	60	2·6	64	56	13
C 14	500	90	1·9	32	25	22
C 15	500	100	0·9	46	37	20
C 16	500	105	2·8	22	17	23
C 17	2000	112	0·8	154	146	6
C 18	1600	121	0·8	188	170	10

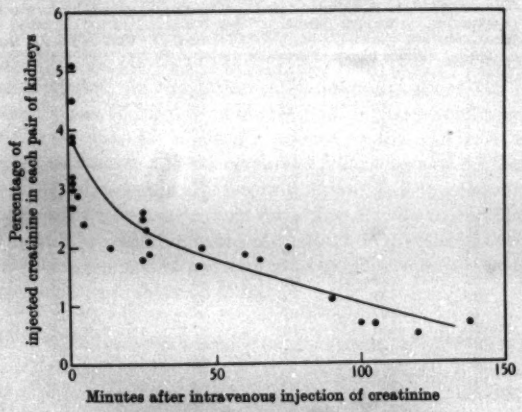

Fig. 1. Amounts of creatinine in kidneys.

The amounts of creatinine in the kidneys.

The amounts of creatinine found in the kidneys in the short sample experiments are of interest in relation to the figures from the long sample experiments. In Fig. 1 the total for each pair of kidneys is given as a percentage of the total amount injected, 0·4 mg. per kidney for pre-existent creatinine being deducted before calculating the percentages. The figures from the long sample experiments and from a few control experiments are also plotted.

It will be seen that the amount of creatinine in the kidneys is at its maximum immediately after the injection of the creatinine and that it then steadily diminishes.

Additional experimental results.

For the purpose of the later discussion of the significance of renal extraction ratios, it is necessary to know what proportion of the injected creatinine is carried in the plasma as compared with that carried in whole blood. This requires consideration of two factors, the relative volumes and the creatinine contents of plasma and corpuscles.

Distribution of creatinine in the blood.

Investigations were made as to the distribution of creatinine between plasma and corpuscles at various times after the intravenous injection.

TABLE VI. Distribution of creatinine between plasma and corpuscles.

Time after injection of creatinine min.	Creatinine mg. per 100 c.c.		Volume of plasma per 100 c.c. blood c.c.	Ratio of amount of creatinine in plasma of 100 c.c. blood to that in 100 c.c. blood p.c.
	Whole blood	Plasma		
1	31	43	70	97
1	122	150	65	80
1	76	95	63	79
1	142	169	65	77
1	58	63	61	67
2	56	59	61	73
2	62	75	54	66
4	52	52	56	56
13	35	32	69	63
13	30	30	55	57
14	117	120	—	—
23	30	27	53	47
25	89	82	60	55
25	54	51	56	54
25	29	26	59	52
26	15	16	57	60
27	29	29	65	65
27	11	11	55	55
101	100	99	62	62
107	67	66	60	58

About 10 c.c. of blood were used. The requisite amount of this blood was taken for analysis, and the rest was spun, at once, on a rapid centrifuge for 3 min.; this yielded more than the 2 c.c. of plasma required for the estimation of its creatinine content. The remainder of the blood was again centrifuged for determinations of corpuscular volume. The results are shown in Table VI. The table may be summarized by certain averages as follows:

Time after injection of creatinine min.	Ratio of amount of creatinine in 100 c.c. plasma to that in 100 c.c. blood p.c.	Ratio of amount of creatinine in plasma of 100 c.c. blood to that in 100 c.c. blood p.c.
1	123	80
3	109	65
25	97	55
105	98	60

Two points need mention. First, it is not possible to say how much diffusion of creatinine occurs between the plasma and corpuscles during the manipulations *in vitro*, such as pipetting and centrifugation. The figures do not therefore give absolutely reliable information about the conditions *in vivo*. As, however, the manipulations *in vitro* were the same for all the bloods, there is clearly some significance in the change of general level of the figures with the lapse of time after the injection. Second, the blood used for the determinations at 1 min. was that from the renal vein. The plasma of this blood had been exposed to the action of the kidney and therefore had lost part of its creatinine; probably it had a rather higher creatinine content, relative to that in whole blood, when it was in the renal artery.

The only legitimate conclusions are as follows. Injected creatinine, which is only in the plasma at the time of the injection, passes gradually into the corpuscles. An equilibrium is established in about 5 or 10 min. with a fairly equal concentration of creatinine in plasma and corpuscles. As the plasma creatinine is steadily reduced by the activity of various organs the concentration in the corpuscles falls in association; but there appears to be some lag in the return diffusion, and the concentration in the corpuscles may thus remain for a time slightly higher than that in the plasma. The absolute amount of creatinine in the blood is eventually distributed between plasma and corpuscles roughly in proportion to their relative volumes; *i.e.* about 60 to 40.

Plasma volumes.

It is convenient at this stage to give the data on which the relative volumes of plasma and corpuscles have been established. In a discussion

in a previous paper [Dunn, Kay and Sheehan, 1931] the corpuscles were assumed to be half the blood volume; the proportion of plasma required to be filtered in the glomeruli to account for the elimination of urea by filtration was therefore taken to be twice the renal extraction ratio. This appears to be erroneous; the present evidence shows that the factor should have been 1·6 instead of 2.

The figures in Table VII were obtained by the thorough centrifugation of about 10 c.c. of blood from each rabbit; the time required for the corpuscles to reach a constant volume was 30–50 min. The blood contained a known amount of 20 p.c. solution of potassium oxalate, in some cases as much as 0·2 c.c., which is sufficient to make the corpuscles shrink to about one-twentieth of their volume [Osgood, 1926]. A correction has been made in each case for the contraction of the corpuscles and also for the volume of oxalate solution added.

Table VII shows the percentage of plasma in:

(*a*) ear vein blood of normal rabbits with no previous injections;

(*b*) ear vein blood of rabbits injected with creatinine solution several minutes previously;

(*c*) heart blood of rabbits injected with urethane solution several minutes previously;

(*d*) heart blood of rabbits injected both with urethane and creatinine solution several minutes previously, and then subjected to short sample experiments;

(*e*) renal vein blood of rabbits injected with urethane solution several minutes previously, and then subjected to long sample experiments involving the injection of creatinine solution intravenously.

TABLE VII. Volume of plasma as percentage of blood volume in individual rabbits.

	Intravenous injection of		Intravenous injection of creatinine and urethane	
Normal	Creatinine	Urethane	Short sample exp.	Long sample exp.
(*a*)	(*b*)	(*c*)	(*d*)	(*e*)
59	50	60	55	61
61	54	61	56	62
62	55	63	57	63
65	56	63	59	63
65	59	63	59	64
74	61	66	59	65
	69	67	60	65
	69		65	66
			66	70
Mean 64·3	59·1	63·3	59·6	64·3

It will be seen that there are very wide variations between different rabbits, but that the average figure is about 62 p.c. The small differences between the means cannot be considered of any particular significance. Even the long sample experiments, where 2 or 5 c.c. of creatinine solution were injected at the beginning of the collection of blood, do not show any definite evidence of dilution of the blood as compared with normals, while the figures of the other groups show a very slightly lower percentage of plasma than the normal.

To investigate fine differences it would clearly be necessary to examine a number of samples of blood from the same rabbit. It appears from investigation of three rabbits which were each bled from the ear on two separate occasions, with a fortnight's interval for examination of the relative amounts of the blood constituents, that the plasma volume is a fairly constant percentage of the blood volume in each individual animal.

The main conclusion to be drawn from these results is that, although the relative volume of the plasma is different in different rabbits, the intravenous injection of urethane and creatinine in the way described in this paper has no recognizable influence on it. This rapid and efficient adjustment of the blood volume is similar to that noted by Smith and Mendel [1920] who, in 2 min., injected 100 c.c. of isotonic solutions of salts intravenously in rabbits and produced only a very transient hydræmia; the blood volume was only raised about 15 p.c. 10 min. later.

UREA.

Long sample experiments.

The renal extraction ratios.

Five experiments are recorded, the details of which are summarized in Table VIII. 25 p.c. urea solution was injected.

TABLE VIII. Urea long sample experiments.

Rabbit	Amount of urea injected g.	Duration of collection of renal vein blood sec.	Amount of urea accumulated in left kidney (observed amount minus allowance of 8·8 mg. for pre-existent) mg.	Total amount of urea in renal vein blood mg.	Renal extraction ratio p.c.
D 1	4·85	40	86	125	41
D 2	5·64	60	57	84	40
D 3	4·88	60	113	275	29
D 4	2·69	60	22	60	26
D 5	3·63	60	24	85	22
					26

The renal extraction ratios lie between 41 and 22 p.c., with a mean of 32 p.c. The significance of these figures will be dealt with later.

The amounts of urea in the kidneys.

The rate of accumulation of urea in the kidneys during the first minute after injection is shown in Table IX.

TABLE IX. Urea long sample experiments.

Rabbit	Mean time of removal of kidneys: sec. after beginning of injection	Amount of injected urea accumulated in kidneys (observed amount minus 17·6 mg. for pre-existent) mg.	Total amount injected mg.	Percentage of total amount injected accumulated in kidneys
D 1	42	183	4850	3·77
D 2	61	143	5640	2·53
D 3	62	223	4880	4·57
D 4	61	45	2690	1·67
D 5	61	46	3630	1·27
Mean	57			2·76

The mean, 2·76 p.c., is decidedly lower than that of creatinine, which was 3·63 p.c. It is nevertheless an indication of rapid accumulation. The average weight of the kidneys was 14·5 g. and that of the animals was 2·26 kg.; the kidneys were therefore 0·64 p.c. of the body weight, but took up over four times this percentage of the injected urea.

The lower proportion of urea taken up as compared with creatinine is probably related to the poorer renal circulation in the urea series at the time of the peak of the blood content. Certainly the renal extraction ratios are slightly higher than in the corresponding creatinine experiments, and it would therefore seem that the kidney is able actually to take up more urea than creatinine if equal amounts of the two substances reach it in the blood.

The blood flow through the kidneys.

The renal circulation rates in these experiments shown in Table X can at best be only a poor indication of the fluctuating blood flows. It is nevertheless rather surprising to find them as high as they are; the blood flow before and after the period of marked vaso-constriction must have been relatively large. Although the blood flow varied so much, it appears to have been the same on both sides. This is shown by the fact that the amounts of urea accumulated in the two kidneys lie within the range

of ± 10 p.c. in each animal and agree satisfactorily on the whole for all animals. D 2 is excluded from this statement; it appears probable that the blood flow was less through its left kidney than through its right.

TABLE X. Urea long sample experiments.

Rabbit	Renal circulation rate c.c. per g. per min.	Total urea in kidneys left/right p.c.
D 1	2·6	90
D 2	(1·2)	(70)
D 3	2·6	103
D 4	1·7	94
D 5	1·6	103
Mean (excluding D 2)	2·11	98

Short sample experiments.

The renal extraction ratios.

The results of the short sample experiments are shown in Table XI, arranged according to the time elapsing between the injection of the urea and the operation. It will be seen that the renal extraction ratios lie between −5 and 8 p.c. A negative extraction ratio means that the renal vein blood contains more urea than the heart blood.

TABLE XI. Urea short sample experiments.

	Urea injected		Renal circulation rate c.c.	Urea mg. per 100 c.c. blood		Renal extraction ratio
Rabbit	mg. per kg.	min. before operation	per g. per min.	Heart	Renal vein	p.c.
E 1	1000	3	0·9	205·0	203·2	1
E 2	1000	3	1·5	206·1	198·7	4
E 3	1000	8	3·0	154·9	144·6	7
E 4	1000	8	3·4	166·2	163·0	2
E 5	540	16	0·9	92·1	96·7	−5
E 6	1000	17	1·9	167·0	164·8	1
E 7	400	17	1·2	47·2	47·8	−1
E 8	1000	18	0·8	171·5	174·0	−1
E 9	950	26	0·8	155·8	163·2	−5
E 10	350	33	1·7	103·7	99·5	4
E 11	1000	35	1·1	160·7	157·0	2
E 12	1050	39	1·3	188·5	188·3	0
E 13	650	45	5·6	116·1	114·7	1
E 14	1710	45	1·3	282·0	277·3	2
E 15	430	46	3·1	80·5	78·3	3
E 16	1820	55	2·8	197·5	188·4	5
E 17	770	70	3·0	137·5	130·0	5
E 18	1000	105	2·3	111·5	106·7	4
E 19	1000	118	3·8	121·1	114·0	6
E 20	1000	147	3·8	132·2	125·1	5
E 21	1000	159	1·9	118·4	108·5	8
E 22	4000	174	4·1	296·2	281·7	5
E 23	1000	189	0·9	80·1	73·5	8
E 24	1000	212	1·2	82·2	77·0	6
E 25	1000	213	3·8	101·2	93·1	8

The figure given for the blood urea content of heart or renal vein blood is the mean of two paired determinations on each sample, agreement between which was usually within 0·3 mg. per 100 c.c.

The blood flow through the kidneys.

In all the short sample experiments the renal circulation rates show the usual wide variations; the average figure is 2·2 c.c. per g. per min. which is about the normal. There is, however, a tendency to low rates for about half an hour after the injection of the urea, and to high rates later. This can be shown by the averages from the short sample experiments at various times after the injection.

Time after injection of urea min.	Number of rabbits	Mean renal circulation rate c.c. per g. per min.
0–25	8	1·7
25–50	7	2·1
50–220	10	2·8

This suggests that the renal vaso-constriction which develops at the time of the injection continues to some extent for several minutes and then gives place to a renal vaso-dilatation. An average renal circulation rate of 2·8 c.c. per g. per min. in any series of ten animals is unusually high and must be regarded as significant. It is of interest to note that Addis [1928] has shown that the kidney function of an animal is at its highest when large amounts of urea and water have been given by mouth some time previously. The renal blood flow appears to be stimulated by this treatment in the same way as it is increased by the intravenous injection of urea about an hour before.

The variations of the renal circulation rates corresponding to the time after the injection of urea are superimposed on the variations which occur in normal animals. This association between the variations is of course not a methodical one; the frequency of occurrence of any particular rate in these experiments is therefore not significantly different from that in normal rabbits. This is seen from the collected results of all the animals from the urea and creatinine experiments, including those discarded on account of poor blood flows or for other reasons. They are shown in Table XII, together with the results of a few similar experiments involving the injection of sodium sulphate. The percentages of animals whose renal circulation rates lay within the individual 1 c.c. ranges detailed below are very close in the present series of 113 measurements to the percentages in a series of 120 experiments which have been recorded previously [Sheehan, 1932*a*]. The mean rate for the sulphate

experiments is rather high; this is in accord with the calculations made by Ekehorn [1931] from the data of Mayrs and Watt as to renal blood flows in rabbits injected with sulphate.

TABLE XII. Renal circulation rates.

Renal circulation rate c.c. per g. per min.	Percentage of measurements in each 1 c.c. range: Present series 113 rabbits	Previous series 120 rabbits
0–1	21	14
1–2	35	41
2–3	22	29
3–4	18	12
4–5	3	3
5–6	1	1

Mean renal circulation rates:

Previous series:	2·0 c.c. per g. per min.
Present series:	
All rabbits	2·1 „ „
53 rabbits injected with urea	2·1 „ „
41 „ „ creatinine	1·9 „ „
19 „ „ sulphate	2·7 „ „

THE RELATIONSHIPS OF THE RENAL EXTRACTION RATIOS.

CREATININE.

Long sample experiments.

The renal extraction ratios lie between 21 and 39 p.c. The range is rather wide and needs some explanation. As in the short sample experiments to be discussed below, the various factors which could possibly influence the ratios have been carefully studied. The only relationships which can be established are:

(1) The highest three renal extraction ratios are those from the shortest three experiments.

(2) There is a tendency to an inverse proportionality between the renal extraction ratio and the renal circulation rate similar to that noticed in the case of dyes during the first minute after injection. This tendency is partly obscured by B 3 which has a high renal circulation rate, but a short time of collection of renal vein blood.

The combined influence of the time of collection and the circulation rate factors is expressed in the volumes of renal vein blood collected. These, as is shown in Table XIII, have an inverse relationship to the renal extraction ratios.

TABLE XIII. Creatinine long sample experiments.

Rabbit	Amount of blood collected from renal vein c.c.	Renal extraction ratio p.c.
B 1	9·9	39
B 2	11·7	32
B 3	13·3	27
B 4	15·2	27
B 5	15·7	26
B 6	15·2	25
B 7	23·0	24
B 8	21·2	21

No definite relationship can be made out between the renal extraction ratio and the actual amount of creatinine coming to the kidney during the experiment. This amount might perhaps be expected to depend on the amount of the blood flow, but such a view is contradicted by the results of the experiments; it does not take into account the differences in the amounts of creatinine injected and the different heights of the peaks of blood creatinine even after the injection of the same amounts of creatinine.

Short sample experiments.

The renal extraction ratios for the short sample experiments are definitely lower than those for the long sample experiments. The fairly wide range of variation which they show among themselves again indicates the need for considering what influence the numerous variable factors in the experiments have on the ratios.

By graphing all the known variable experimental factors against the renal extraction ratios, two were established as significant, and will later be discussed fully. With these in mind, the others were re-examined, but with negative results, and are here enumerated without elaboration.

(1) Weight, sex, fatness, calculated blood volume of animal.

(2) Amount and dosage of creatinine injected.

(3) Volumes of solutions of creatinine and urethane injected.

(4) Dose of urethane; time between its injection and operation; depth of anæsthesia. (These were fairly constant.)

(5) Amount of operative interference. (Fairly constant. Where the collection of renal vein blood was unduly prolonged, the experiments were discarded.)

(6) Renal circulation rates. The suggestion of a direct relationship between these and the extraction ratios, especially at high and low values, is not conclusively established by these experiments.

The two significant factors are to some extent inter-related. They are:

(*a*) the time elapsing between the injection of the creatinine and the operation;

(*b*) the amount of creatinine in the heart blood.

On graphing the renal extraction ratios against the time after the injection a very definite relationship can be recognized. The ratios during the first minute, as ascertained by the long sample experiments, are usually about 27 p.c. After this, during the first 20 min., there is a marked tendency to low ratios. From 25 min. onwards the ratios rise

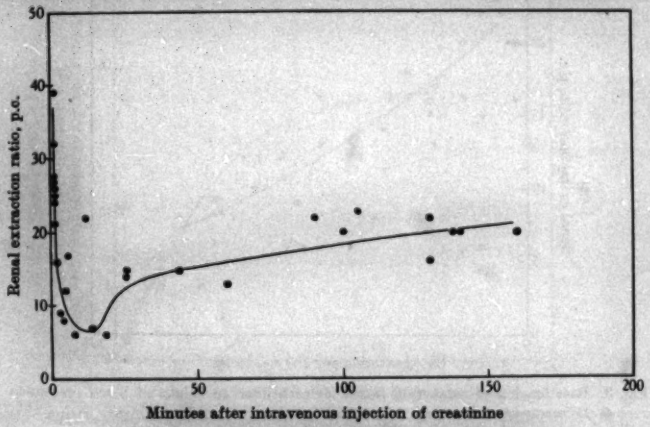

Fig. 2. Relationship of renal extraction ratios for creatinine to time after injection.

steadily to about 20 p.c. This is shown in Fig. 2. Exps. C 17 and C 18 are not included in this Figure as they are essentially examples of the action of the second factor. The experiments of series F are, however, comparable, and are included.

The extraction ratios during the first minute have been discussed earlier. The question that now arises is why there is the dip in the curve at 6–24 min. after the injection. Is this phenomenon related to the time interval alone, or is it the result of some other concomitant factor?

The key to the problem lies in the figures after 24 min. These ratios are from a time when the interfering factor of the dip in the curve has passed off.

These experiments, C 10–C 18, are graphed in Fig. 3 against the height of the blood creatinine. The figures of ten further short sample experiments of series F and G are also included; these are experiments about 2 hours after the administration of the creatinine, and the "time dip" is thus not a complicating factor. It is clear from the Figure that the renal extraction ratios tend to an inverse relationship with the height of the blood creatinine.

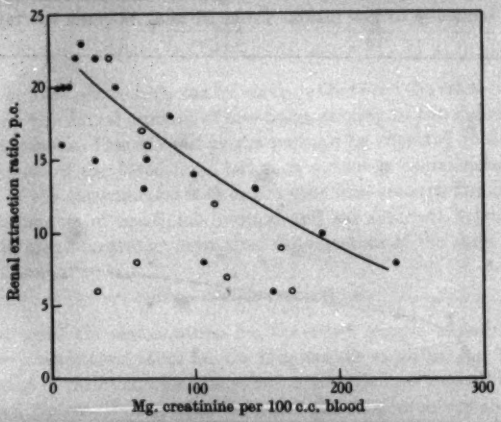

Fig. 3. Relationship of extraction ratios for creatinine to height of blood creatinine. ● Experiments later than 24 min. after injection. ○ Experiments earlier than 24 min. after injection.

The Figure also shows by a different notation the renal extraction ratios of C 1–C 9, *i.e.* less than 24 min. after injection. It will be seen that in five cases the low ratios at this time are satisfactorily explained by the height of the blood creatinine; of the other four ratios, two are low and two are unusually low. The results of the urea experiments are of assistance in suggesting an explanation for these four low ratios during the first 24 min. after injection.

The relatively lower efficiency of the kidney in dealing with high blood creatinines than with small ones is of decided interest in relation to Marshall's recent work on the plasma clearance of phenol red in dogs [1931]. This author showed that the kidney is relatively rather less efficient in secreting this dye from high blood concentrations than from low ones.

The point may also be demonstrated by comparing the actual differences between the creatinine content of the heart blood and renal vein blood at different levels of the blood creatinine. Fig. 4 shows that these "differences" increase with the height of the blood creatinine, but at a diminishing rate. The gradual diminution of the renal extraction ratios shown in Fig. 3 is a measure of the failure of the kidney to maintain a strictly proportional increase in the "differences" shown in Fig. 4. From the graph-points for blood creatinines of 20–40 mg. per 100 c.c.

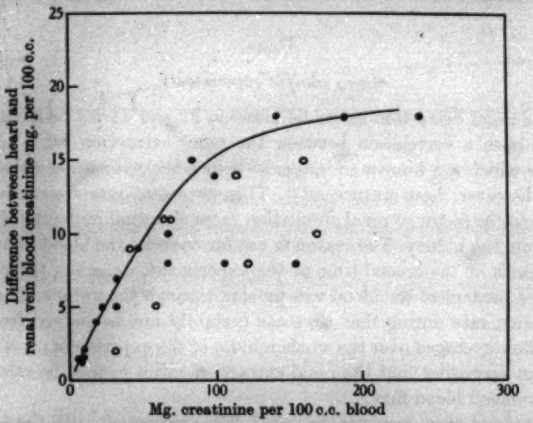

Fig. 4. Relationship of difference between heart and renal vein blood creatinine to height of blood creatinine. ● Experiments later than 24 min. after injection. ○ Experiments earlier than 24 min. after injection.

it might be expected that, if the proportion remained constant, the "difference" would be 25 mg. per 100 c.c. at a blood creatinine of 120 mg. per 100 c.c. Actually the figure is about 17 mg. per 100 c.c. It appears doubtful whether the "difference" in these short sample experiments would ever reach 25 mg. per 100 c.c., no matter how high the blood creatinine were raised. This, however, is only a matter of speculation; the blood creatinine has not been higher than 250 mg. per 100 c.c. in any of the short sample experiments.

It will also be seen from the Figure that the four experiments before 24 min., where the renal extraction ratios were low, have also an unusually low "difference."

This part of the discussion may be summarized in two conclusions:

(1) The difference between the creatinine content of heart blood and renal vein blood increases as the creatinine content of the heart blood rises, but in a steadily diminishing proportion. As a result, there is an inverse relationship between the renal extraction ratio and the height of the blood creatinine.

(2) From 6 to 24 min. after the injection of the creatinine the renal extraction ratio is sometimes lower than would be expected from the relationship of renal extraction ratios to blood creatines at other times.

Urea.

Long sample experiments.

The renal extraction ratios lie between 22 and 41 p.c. All attempts to establish a correlation between the renal extraction ratio and the factors which are known to influence it in work with other substances have, however, been unsuccessful. This statement refers essentially of course to the factor of renal circulation rates or actual amounts of blood flow from the kidney. The reason is not far to seek: the blood flows were so variable at the crucial time of the experiment, *i.e.* when the peak of the urea content of the blood was passing through the kidney. The renal circulation rate during that time can certainly not be judged from the blood flow averaged over the whole minute of the experiment; it is therefore not surprising that the renal extraction ratios cannot be related to this averaged blood flow.

One point may, however, be noted. The experiment with the highest renal extraction ratio is that in which the renal vein blood was collected for the shortest time. The same phenomenon was observed in the long sample experiments with creatinine.

Short sample experiments.

The experiments of series F are included in this discussion as they show no differences from those of series E. The renal extraction ratios for these raised blood ureas are very variable; they lie between −5 and +8 p.c.

As with creatinine, they have been graphed against all observed factors; again the two important factors are the time elapsing between the injection of the urea and the operation, and the amount of urea in the heart blood. The time relationship is considered first; it is shown in Fig. 5.

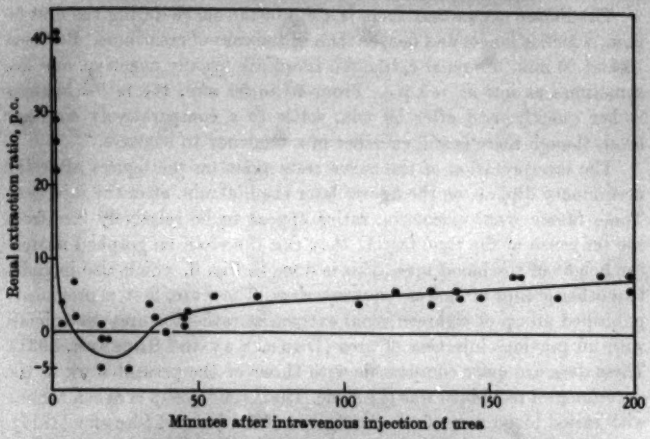

Fig. 5. Relationship of renal extraction ratios for urea to time after injection.

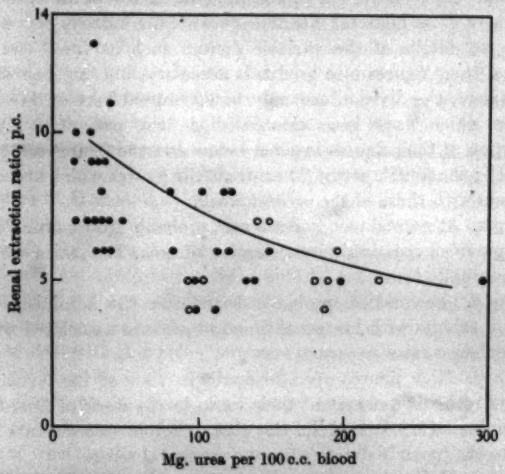

Fig. 6. Relationship of renal extraction ratios for urea to height of blood urea.
● Authors' figures. ○ Figures of Addis and Shevky.

The Figure shows that there is a dip in the curve during the first 50 min., which is longer and deeper than in the case of creatinine. Between 16 and 26 min. the renal extraction ratios are usually negative, and are sometimes as low as −5 p.c. From 40 to 50 min. the ratios increase rather quickly, and after 50 min. settle to a comparatively constant level, though there is still evidence of a tendency to increase.

The interpretation of the curve rests again on the figures after the preliminary dip, *i.e.* on the figures later than 50 min. after the injection. These fifteen renal extraction ratios appear to be relatively free from the influence of the time factor; they can therefore be graphed against the height of the blood urea. This is done in Fig. 6, which also includes two other groups of figures for comparison. These are, first, a previously published group of eighteen renal extraction ratios for urea on animals with no previous injection of urea [Dunn, Kay and Sheehan, 1931]. These data are quite comparable with those of the present work as the experimental technique was the same. The second group is of ten rabbits with raised blood ureas from the figures of Addis and Shevky [1917]; this group is shown in the Figure by a distinctive sign.

Addis and Shevky employed a technique roughly similar to the short sample experiment. They publish, in all, figures of nineteen experiments where gross bilateral interference with the kidneys was avoided, but give no details of the variable factors such as time, etc. Some division of these figures into groups is necessary, but, as their data are so incomplete, the division can only be attempted here on the general principles which have been established in the present study. This classification of their figures is given below and the figures are given in Table XIV; the fourth group (δ) contains the figures which are used for comparison with those of the present work.

Group α. At normal blood urea levels, probably from normal animals with no previous or recent administration of urea. The ratios are nevertheless unusually low.

Group β. Low ratios, probably soon after the administration of urea, or in rabbits with low renal function perhaps associated with low renal circulation rates.

Group γ. High figures open to doubt in view of the levels of the extraction ratios in the rest of their work, in the work of Dunn, Kay and Sheehan [1931], and in the short sample experiments of the present work; possibly due to some experimental error.

Group δ. Raised blood ureas and satisfactorily functioning kidneys, probably some time after administration of urea.

TABLE XIV. Results of Addis and Shvcky's experiments.

Group	Urea mg. per 100 c.c. blood		Renal extraction ratio
	Jugular vein	Renal vein	p.c.
α	23	22	5
	27	27	0
	38	38	0
β	78	77	1
	129	130	−1
	197	196	0
	216	211	2
γ	53	42	21
	57	47	18
δ	93	88	5
	96	92	4
	102	97	5
	140	130	7
	149	139	7
	178	169	5
	187	180	4
	189	180	5
	196	184	6
	223	212	5

To return to Fig. 6. There is a reasonable agreement of the present results with the group δ figures of Addis and Shevky. It will be seen that the renal extraction ratio is in general lower as the blood urea increases. The inverse relationship between the efficiency of the kidney and the height of the blood urea is a similar phenomenon to that observed in the case of creatinine. It can also be demonstrated by plotting the actual differences between the urea content of renal vein blood and heart blood against the height of the blood urea as in Fig. 7.

The same two groups of figures are again included for comparison; the high blood urea figures of Addis and Shevky and the normal blood urea figures of Dunn, Kay and Sheehan. The gradual failure of the kidney to keep the "difference" proportional to the blood urea is seen quite clearly. It seems possible from extrapolation that the maximum "difference" that could be reached would be between 15 and 20 mg. per 100 c.c. at a blood urea of about 500 mg. per 100 c.c. This is of course quite hypothetical; as mentioned earlier a number of experiments were planned to deal with this point, but were unsuccessful owing to the toxic effect of the large doses of urea required.

With regard to the group of experiments performed earlier than 50 min. after the injection of the urea, consideration must be given to the question of whether the low renal extraction ratios are indicative merely of the height of the blood urea at the time. By comparison of the ratios

of E 1–E 15 (from the "dip" in the time graph) with the ratios plotted in Fig. 6 it will be seen that E 2 and E 3 gave renal extraction ratios which were normal at their levels of blood urea, that E 10 gave a ratio a little lower than the normal range, and that all the remainder are very definitely subnormal. It is quite clear that the amount of time elapsing after the injection is a factor of great importance during the first 50 min., and that the height of the blood urea is not of the same significance at that time.

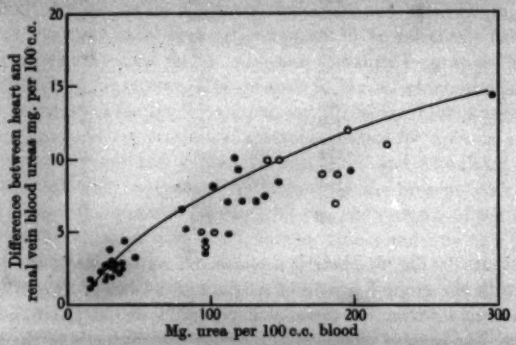

Fig. 7. Relationship of difference between heart and renal vein blood ureas to height of blood urea. ● Authors' figures. ○ Figures of Addis and Shevky.

The conclusions to be drawn from the short sample experiments about urea are almost identical with those already drawn about creatinine.

(1) The difference between the urea content of heart blood and renal vein blood increases as the urea content of the heart blood rises, but in a steadily diminishing proportion. As a result, there is an inverse relationship between the renal extraction ratio and the height of the blood urea.

(2) From 6 to 50 min. after the injection of the urea the renal extraction ratio is almost invariably lower than would be expected from the relationship of renal extraction ratios to blood ureas at other times.

COMBINED UREA AND CREATININE EXPERIMENTS.

Having ascertained the two important factors which affect the renal extraction ratios in the cases of creatinine and urea, it is now possible to investigate the ratios for both substances when they are injected together in the same animal. Such an experiment should be performed at an interval after the injection of the urea and creatinine sufficient

to avoid the complicating factor of the "dip" period. The only known factor remaining is the amount of the substance in the blood, and the influence of this factor can be estimated sufficiently closely from Figs. 3 and 6. It is thus possible to find whether the administration of either creatinine or urea interferes with the renal extraction ratio for the other substance.

The purpose of the first series of combined experiments was to raise the blood urea fairly high, but to raise the blood creatinine only sufficiently to allow of accurate estimation: then, by short sample experiment, to find the renal extraction ratios for the two substances, and to compare the ratios with those obtained when each substance is injected singly.

A mixed solution of urea and creatinine was injected intravenously in each rabbit; the doses were always 1000 mg. of urea and 250 mg. of creatinine per kg. The operations were performed at 130–160 min. later. The renal circulation rates were normal, varying between 1·6 and 3·1 c.c. per g. per min. with an average of 2·3 c.c. per g. per min. The blood ureas were about 100 mg. per 100 c.c., the blood creatinines about 10 mg. per 100 c.c. At these levels it will be seen from Figs. 3 and 6 that the renal extraction ratios are about 6 p.c. for urea and 20 p.c. for creatinine when the substances are given singly.

The chief results of the experiments are given as series F in Table XV. It will be seen that the renal extraction ratios lie within the range found when the substances are injected singly. The conclusions from this series of experiments are that a fairly large elevation of the blood urea does not affect the renal extraction ratio for creatinine, and that a small elevation of the blood creatinine does not affect the renal extraction ratio for urea.

A second series of experiments was then performed to test the effect of raising the blood creatinine to high levels while leaving the blood urea

TABLE XV. Combined experiments.

	Urea mg. per 100 c.c. blood		Creatinine mg. per 100 c.c. blood		Renal extraction ratio p.c.	
Rabbit	Heart	Renal vein	Heart	Renal vein	Urea	Creatinine
Large doses of urea:						
F 1	147·6	139·2	18·0	14·0	6	22
F 2	95·7	92·1	8·2	6·9	4	16
F 3	111·2	104·1	9·3	7·5	6	20
F 4	95·9	91·5	7·1	5·7	5	20
F 5	119·9	110·6	10·4	8·3	8	20
Large doses of creatinine:						
G 1	38·1	37·9	240	222	0	8
G 2	50·1	48·9	141	123	2	13
G 3	48·4	48·4	105	97	0	8
G 4	24·9	24·3	84	69	2	18

low. These rabbits received creatinine intravenously in doses from 1·6 to 2·0 g. per kg.; they were given no urea. The short sample experiments were performed at 112–131 min. after injection. The renal circulation rates were low, ranging from 0·8 to 1·2 c.c. per g. per min., with an average of 0·9 c.c. per g. per min. The blood creatinines were raised to 84–240 mg. per 100 c.c. From Fig. 3 it will be seen that at these levels the usual renal extraction ratios for creatinine are about 16 and 8 p.c. respectively.

The results of the experiments are given as series G in Table XV. The renal extraction ratios for creatinine are within the range found in experiments where creatinine was investigated alone. The renal extraction ratios for urea are, however, somewhat surprising. The blood ureas were normal and the renal extraction ratios would therefore be expected to lie between 6 and 13 p.c. [Dunn, Kay and Sheehan, 1931]. They lie in the present experiments at 0 and 2 p.c. instead. There is clearly a marked impairment of urea elimination.

The results of the two series of experiments may be illustrated in another way. The average differences between heart blood and renal vein blood are as follows:

	Difference Heart blood—renal vein blood mg. per 100 c.c.	
	Urea	Creatinine
Series F	6·6	2·3
Series G	0·5	15·0
Normal rabbits	2·5	0·15*

* Estimated from creatinine excretion in urine.

In series F, in which large amounts of urea were injected, the kidneys were removing absolutely much more urea and creatinine from each 100 c.c. of blood than in the normal rabbit, though the renal extraction ratio for urea was of course lower than in rabbits with normal blood urea.

In series G, in which large amounts of creatinine were injected, there was not only a relative reduction of urea elimination, as expressed in the renal extraction ratios, but an absolute reduction as well. The kidneys were removing only about a fifth of the urea that is removed from each 100 c.c. of blood in normal rabbits. This specific interference with urea elimination is of interest. There was also an interference with the renal circulation, the blood flow being only about half the normal. (See also the comparable experiments, C 17 and C 18 in Table V.) But with regard to creatinine the kidneys were working excellently and taking 15 mg. of this substance from every 100 c.c. of blood; it can thus not be considered that there was any general depression of renal function.

The interference with the renal extraction ratios for urea leads, as would be expected, to some elevation of the blood urea. In five rabbits the blood urea was measured immediately before and at 4 hours after an intravenous injection of 2000 mg. of creatinine per kg.; the dose was the same as that used in series G. The results are given in Table XVI; it will be seen that there is an average increase of nearly 10 mg. urea per 100 c.c. at 4 hours after administering the creatinine.

TABLE XVI. Effect of large doses of creatinine on blood urea.

	Blood urea mg. per 100 c.c.	
Rabbit	Before creatinine injection	4 hours after creatinine injection
H 1	29·4	37·6
H 2	38·9	51·2
H 3	39·4	50·2
H 4	48·2	55·0
H 5	48·4	58·2
Mean	40·8	50·5

PLASMA RENAL EXTRACTION RATIOS.

CREATININE.

The discussion so far has taken no account of the relative amounts of creatinine in the plasma and in the corpuscles. For the purpose of simplicity all renal extraction ratios have been calculated as those for whole blood. It is now necessary to consider a complicating factor of definite and fundamental importance.

The creatinine in the blood is not carried in the plasma alone. Part of it is in the corpuscles; presumably it has entered them by a process of diffusion. For practical purposes the creatinine in the corpuscles is outside the immediate scope of the activity of the kidney. This follows from the following considerations. The initial diffusion from the plasma to corpuscles is not very rapid; at 1 min. after the injection the corpuscles contain only 20 p.c. of the total creatinine in the blood, though later they will contain 40 p.c. During the first minute the conditions favouring diffusion are very good, the plasma having a high creatinine content and the corpuscles a very low one. This may be contrasted with the condition of the blood which is exposed to the action of the kidney a few minutes later. At that time an equilibrium has been reached with the percentage of creatinine in the plasma about equal to that in the corpuscles. If the kidney suddenly removes 20 p.c. of the creatinine in the plasma passing through the renal capillaries, the equilibrium will be disturbed and a

return diffusion of creatinine from corpuscles to plasma will take place. It is, however, most unlikely that such a return diffusion can reach any significant proportion during the 3 or 4 sec. that the blood is passing through the renal capillaries, since the known rate of diffusion during 60 sec. is, as shown above, not really very great even under very favourable conditions. Probably, therefore, the creatinine in the corpuscles passes through the renal capillaries practically unaltered in amount.

The significant figure in considering the renal elimination of creatinine is, therefore, the amount of creatinine in the plasma going to the kidney, since only this amount of creatinine is exposed to the activity of the kidney. The figures given in an earlier section may be summarized as follows. At the time of the injection the plasma contains practically all the creatinine in the whole blood; at the end of 1 min. it contains 80 p.c. of it. An average figure of at least 90 p.c. in the plasma may be taken as representing approximately the condition during the first minute. On the other hand, after the first minute the plasma contains 60 p.c. of the creatinine in the whole blood.

The bearing of these points on the renal extraction ratios is that the kidney is not taking up creatinine from the whole amount in the blood but only from the part of it in the plasma. The conception of a "plasma renal extraction ratio" must be added to that of the ordinary "whole blood renal extraction ratio." The plasma renal extraction ratio is the true measure of renal elimination, since the ordinary ratio includes creatinine which is not exposed to the activity of the kidney. The calculation of the plasma renal extraction ratio for any individual experiment depends of course on the proportion of creatinine in the plasma having been ascertained exactly, but the average ratios for the whole series of experiments can be calculated with sufficient accuracy by multiplying the ordinary ratios during the first minute by 100/90 and those after the first minute by 100/60.

Without going into the figures of individual experiments the following averages may be given:

	Whole blood renal extraction ratio p.c.	Plasma renal extraction ratio p.c.
During first minute after injection	28	31
More than 24 min. after injection:		
(*a*) At blood creatinine of about 20 mg. per 100 c.c.	23	38
(*b*) At blood creatinine of about 100 mg. per 100 c.c.	14	23
(*c*) At blood creatinine of about 200 mg. per 100 c.c.	8	13

It should be explained at this point that the distribution of creatinine in the blood between plasma and corpuscles has not been studied in every experiment. For this reason the matter has not been raised earlier, since it would have involved expressing certain results as plasma renal extraction ratios and others as whole blood renal extraction ratios. The general level of the plasma renal extraction ratios can, however, be established quite satisfactorily by the method here adopted. In the individual experiments where the actual plasma-corpuscle proportion of creatinine was measured, the plasma renal extraction ratios are in agreement with the figures calculated for the whole series of experiments.

Urea.

Unfortunately no actual measurements were made of the distribution of urea between corpuscles and plasma in the present work; the amount of apparatus required made such additional investigations impracticable. Certain conclusions, however, may legitimately be drawn from the facts which are known.

It has been established by many workers that the urea content of the corpuscles is the same as that of the plasma, whatever the level of the blood urea. Thus at several minutes after the injection of urea the same condition may be presumed to obtain; the plasma therefore contains 60 p.c. of the whole amount of urea in the blood. The only question which requires more detailed consideration is the rate at which this equilibrium is reached. Some indirect information on the point can be obtained from a comparison of the rates of disappearance of urea and creatinine from the heart blood. The following figures represent the approximate amounts in the heart blood at two intervals after the intravenous injection of 1000 mg. of the substance per kg.

	Urea mg. per 100 c.c. blood	Creatinine mg. per 100 c.c. blood
5 min. after injection	170	280
50 „ „	140	140

It will be seen that both substances reach the same level in the blood after 50 min., but that the initial rate of disappearance of urea is much greater. The disappearance is due chiefly to the passage of the substance into various tissues of the body. It would therefore appear that urea can pass more readily from the blood into the tissues than creatinine. Probably the same rule applies to the passage from plasma to corpuscles. It seems therefore permissible to assume that the mean proportion is 80 p.c. for urea as against 90 p.c. for creatinine. As indicated above,

a proportion of 60 p.c. of the urea in the plasma can be accepted as quite accurate for experiments several minutes after the injection.

The following plasma renal extraction ratios are calculated by multiplying the average ordinary renal extraction ratios during the first minute by 100/80 and those after the first minute by 100/60.

	Whole blood renal extraction ratio p.c.	Plasma renal extraction ratio p.c.
During first minute after injection	32	40
More than 50 min. after injection:		
(*a*) At blood urea of about 30 mg. per 100 c.c.	9	15
(*b*) At blood urea of about 100 mg. per 100 c.c.	7	12
(*c*) At blood urea of about 200 mg. per 100 c.c.	5	8

[(*a*) includes the figures of Dunn, Kay and Sheehan on normal rabbits.]

INTERPRETATION OF THE RENAL EXTRACTION RATIOS.

To avoid misunderstanding it is necessary to define certain words used in this section. *Reabsorption* means the passage of a substance from tubular lumen to tubular epithelium; *direct absorption* means the passage from the blood in the intertubular capillaries to the tubular epithelium. *Secondary return* means the passage from tubular epithelium to the blood in the intertubular capillaries; *secretion* means the passage from tubular epithelium to tubular lumen. A substance may enter the tubular epithelium by either of the first two methods, and leave it by either of the second two methods; *e.g.* there is no fundamental reason why direct absorption should be followed by secretion rather than by secondary return. Furthermore, a substance entering the tubular epithelium by either method may leave it at once, or only after an interval, or may be stored or metabolized there. Of course, none of these processes comes into play in the case of a substance which is eliminated from the blood by filtration alone and which passes down the tubule without any reabsorption.

The present work deals merely with the elimination of urea and creatinine from the blood by the kidney. This is not to be confused with the excretion of these substances in the urine. The two processes must finally balance; there is certainly not a permanent accumulation of the substances in the kidney. But there is no evidence that each unit of urea or creatinine removed by the kidney from the blood is at once excreted into the urine. The significance of this distinction has been the subject of discussion elsewhere [Ekehorn, 1932; Sheehan, 1932 *b*, 1933].

It may be mentioned at this point that, though the kidney may possibly produce or utilize creatinine or urea, such processes can hardly play any significant part in the present experiments, in view of the relatively large amounts of the two substances which have been administered.

The essential results of the whole investigation are summarized below. The figures given are arithmetical averages, but they are given here only because they indicate the general level of the ratios. Any single experimental result represents only a brief incident in the renal activity of one animal; yet, taken all together, the results obtained from many animals under different experimental conditions as to time, plasma concentration, etc. may reasonably be considered to indicate the usual renal response of any animal to the particular substance injected. The results are calculated to give the proportion of the substance which is removed from the plasma during its passage through the kidney.

	Plasma renal extraction ratio p.c.	
	Urea	Creatinine
During first minute after injection	40 (maximum 50)	31 (maximum 43)
At 2–20 min. after injection	2	19
More than 50 min. after injection:		
(*a*) At blood content of about 20 mg. per 100 c.c.	15	38
(*b*) At blood content of about 100 mg. per 100 c.c.	12	23
(*c*) At blood content of about 200 mg. per 100 c.c.	8	13

The next step is to consider how these figures are to be interpreted in terms of renal function.

Ratios later than 50 min. after the injection.

The chief problem that arises is the explanation of the inverse relationship between the amount of creatinine or urea in the blood and the renal extraction ratios for these substances, in the experiments after the early "dip" in the graphs has passed off. Associated with this is the problem of the renal extraction ratios found in the combined experiments.

The former question will be considered first in the case of each substance.

Creatinine.

The "filtration" theory affords two possible explanations, (*a*) decreased filtration, or (*b*) increased reabsorption and secondary return at high blood creatinines.

Decreased filtration.

It is exceedingly improbable that the large amount of creatinine in the blood could provide any physical impediment to filtration of the type exerted by proteins. The only reasonable theory is that the high blood creatinine interferes with filtration by producing vasomotor effects on the glomerular arterioles or capillaries. Although this need not necessarily be associated with any alteration of renal blood flow, it is of interest to note the low renal blood flows after the administration of very large amounts of creatinine in C 17 and C 18 and G 1–G 4. This vasomotor disturbance is not directly related to the height of the blood creatinine as is seen from experiments C 1–C 15. Nevertheless, the occurrence of vasomotor effects in the former cases makes the occurrence of other types of vasomotor effects a distinct possibility.

This theory provides, however, no plausible reason for the almost complete arrest of urea elimination in series G.

Increased reabsorption and secondary return.

At first sight it appears rather paradoxical that reabsorption and secondary return should increase when the blood creatinine is very high, and therefore when the animal is in most need of excreting the substance. Two points must, however, be borne in mind:

(1) The increased reabsorption and secondary return are only relative. The absolute difference between the creatinine in heart blood and renal vein blood does become greater as the blood creatinine rises; the problem at issue is why this difference becomes a steadily diminishing proportion of the blood creatinine.

(2) Reabsorption and secondary return are not necessarily active functions of the tubular epithelium; they may represent only a passive diffusion from the tubular lumen back to the blood. Now there is a maximum concentrating power of the kidney as a whole for different substances; *e.g.* from estimations on bladder urine it is found that the rabbit concentrates urea only up to about 5 p.c., the cat up to about 10 p.c. There is presumably therefore a maximum concentration in the renal tubules which is specific for each substance. Information as to the exact level of this maximum concentration in the different parts of the tubule is unobtainable. But when the blood creatinine is very high a relatively smaller removal of water from the filtrate may raise the creatinine in the tubular urine to near its maximum concentration. In this case the tubular epithelium will be under steadily increasing strain. It may respond either by reabsorption and secondary return of creatinine

(either as an active process or merely by allowing diffusion) or by removing less water from the tubular urine. The former would provide a reasonable explanation of increasing reabsorption and secondary return with increase of the blood creatinine and thus for a diminishing renal extraction ratio.

Furthermore, if the tubular epithelium is under heavy strain in dealing with creatinine, it seems possible that it might fail also to retain urea efficiently in the tubule, even though the urea was not in high concentration.

According to the "secretion" theory it would be expected that the tubular epithelium would be stimulated to absorb directly and secrete a greater absolute amount of creatinine the higher the blood creatinine. In opposition to this, the greater the absolute amount of creatinine secreted the greater would be the work done by the tubular epithelium, and therefore presumably the less efficiently would this epithelium deal with additional work. In this connection, the available supply of oxygen might be a factor of importance, but this is still undecided. Gremels [1930] found that the oxygen usage of the kidney is roughly proportional to the amount of excretion of nitrogenous substances; on the other hand Rhoads, van Slyke, Hiller and Alving [1931] found that the oxygen consumption of the kidney is not markedly affected by increasing the urea excretion.

If the tubular epithelium were under heavy load in directly absorbing and secreting creatinine, it is of course quite possible that it would be unable to deal with urea efficiently, and thus that the renal extraction ratio for urea would be lowered when the blood creatinine was very high, as in series G.

Urea.

The same explanations apply to the urea results as to the creatinine results.

The sudden reduction of renal blood flow during the injection in the long sample experiments is evidence that urea can produce vasomotor disturbance. The possibility thus arises that it may affect the vasomotor control of filtration in all the experiments. This, however, does not explain the fact that the renal extraction ratios for creatinine in series F were not affected by the high blood urea. If the amount of filtration were the controlling factor, a reduction in the percentage of plasma filtered should affect the renal extraction ratio of the two substances equally.

The occurrence of increased reabsorption and secondary return when the blood urea is high would provide a more satisfactory explanation.

The fact that the renal extraction ratio for urea is lower than that for creatinine means, according to a pure "filtration" theory, that much of the filtered urea is normally reabsorbed into tubular epithelium and secondarily returned to the blood. This process may be presumed to occur fairly easily, and apparently is capable of increase without necessarily involving the reabsorption and secondary return of creatinine.

On the other hand, a "secretion" theory offers a plausible explanation. As in the case of creatinine, the gradual diminution of the renal extraction ratios with rising blood urea is to be expected, as the work done increases with increase of the absolute elimination of urea from each 100 c.c. of blood. An easy solution can also be given for the problem of series F and G.

By reference to the average "differences" in these two series tabulated earlier, it will be seen that in series F the absolute amount of urea eliminated from the blood by the kidney was about 4 mg. per 100 c.c. more than normal. This cannot be considered a serious overload for the kidney which has normally to eliminate 2·5 mg. of urea from each 100 c.c. of blood and can remove as much as 15 mg. per 100 c.c. The kidney was also able to eliminate at the same time over 2 mg. per 100 c.c. more creatinine than normal without any evidence of failure.

On the other hand, in series G the kidney was eliminating about 15 mg. creatinine more than usual from each 100 c.c. blood, which is about a hundred times the normal amount. This may be considered to be probably a big load for the tubular epithelium, the highest "difference" observed in any experiment being 18 mg. per 100 c.c. With this work to be done in dealing with creatinine, it is hardly surprising that the kidney practically ceased work with regard to urea.

The inverse relationship between the height of the blood urea or creatinine and the renal extraction ratio for these substances can thus be explained sufficiently by either a "filtration" theory or a "secretion" theory. From the point of view of the former theory, it appears more probable that reabsorption and secondary return are increased than that filtration is diminished when the blood urea or creatinine are raised.

The results of the experiments where both substances were injected together can also be explained by either theory, but are more readily explainable on the view that the elimination of the substances is an active process.

RATIOS DURING THE FIRST MINUTE AFTER THE INJECTION.

The ratios for creatinine and urea found in the long sample experiments can be explained fairly easily by a "filtration" theory. It is necessary to assume that about 30–40 p.c. of the plasma is filtered in the glomeruli and that, even if reabsorption of urea or creatinine into tubular epithelium does occur during the first minute, there is no secondary return of the substances to the blood during this time. The plasma renal extraction ratios are thus about maximal, and give a direct measure of the percentage of plasma filtered. The fact that the ratios for urea are higher than those for creatinine during the first minute is explicable on the view that the vasomotor disturbance produced by the injection of the urea may increase the amount of filtration to as much as 50 p.c. of the plasma entering the renal blood vessels.

The ratios can, however, be equally well explained as due to the direct absorption of creatinine and urea from the blood by the tubular epithelium. This epithelium contains a relatively small amount of the substance at the start of the experiment. Suddenly the blood in the renal capillaries comes to contain a large amount of the substance. It seems not unlikely that the tubular epithelium should take up the creatinine or urea rapidly until a state of equilibrium is reached between the epithelium and the blood. This direct absorption may be either an active process on the part of the cells or merely diffusion of the substance, or both.

The view that the kidney absorbs creatinine and urea directly from the blood during the first minute is supported by two facts. First, the tubule cells certainly present to the blood stream a surface capable of direct absorption, as has been shown previously in the case of dyes. Second, the urea and creatinine are removed from the blood by many other tissues during the first minute after injection, as is obvious from Figs. 8 and 9 (which are given later); this can only be by direct absorption of the urea and creatinine by these tissues. The kidneys take up a bigger proportionate amount of these substances than the average for the rest of the body. There do not seem to be satisfactory grounds for denying to the kidneys a property which is possessed by most other tissues in the body excluding fat and bones [Marshall and Davis, 1914].

Comparison of these two figures shows also that urea is removed from the general blood stream more rapidly during the first few minutes than is creatinine. The higher renal extraction ratio for urea than for creatinine indicates that the kidney is, like the other tissues of the body,

able to remove urea more easily than creatinine from the blood during the first minute after injection. A similarity in the method of removal of the substances by the kidney and by other tissues is certainly suggested.

The further question arises as to why the ratios found in the long sample experiments tend to be higher when the collection of renal vein blood is short than when it is long. On the basis of a "filtration" theory, if the amount of filtration remains fairly constant, it is necessary to assume a rather marked loss of creatinine (or urea) from the kidney in the second half minute during which renal vein blood is being collected. Such loss might be by excretion into the bladder, or by reabsorption and secondary return to the blood stream. As is discussed later, it seems unlikely that any significant loss by excretion can occur; the possibility that reabsorption and secondary return may be in progress remains.

On the other hand, the facts can be easily explained on the view that the early rapid accumulation of creatinine occurs by direct absorption. During the initial peak of high creatinine in the blood the kidney takes up a great deal of creatinine from the blood. There is then a sudden fall in the creatinine content of the blood. The conditions which led to the initial rapid direct absorption are thus reversed in the latter half minute of the blood collection, and it is therefore to be expected that direct absorption will be much reduced or perhaps even replaced by secondary return.

The same explanations which have been given for the creatinine ratios apply of course equally to urea.

Ratios at 2 to 20 min. after the injection.

The dip in the extraction curves of both urea and creatinine during the first 20 min. follows on the rapid taking up of these substances by the kidney, and occurs at a time when their concentration in the blood has fallen much below its peak level. According to either theory a negative extraction ratio is undoubted evidence of secondary return. In the urea experiments there is therefore proof that urea sometimes passes from the tubular epithelium into the blood. This cannot be interpreted as indicating by which route this urea had entered the tubular epithelium. Rhoads, van Slyke, Hiller and Alving [1931] remark that "the occurrence of renal blood urea contents markedly higher at times than arterial urea contents appears to be proof of the occurrence of reabsorption of urea from the kidneys." Their conclusion is quite justified, so long as their term "reabsorption" is understood to refer only to what

has been called here "secondary return" of urea from the kidneys to the blood. It must obviously not be extended to include "reabsorption" of urea from tubular urine into the kidney parenchyma.

Explained by a "filtration theory," a negative extraction ratio would indicate that more urea is being reabsorbed and secondarily returned to the blood than is being removed from the blood by filtration. In this connection two points must be recognized. First, the time required for reabsorbed urea to pass across the epithelial cells and be returned to the blood is quite unknown; the process may be almost immediate or take a long time. Second, the tubular urine contains much urea which could act as a supply for reabsorption and secondary return apart from urea in the fluid which is actually being filtered during the time of the experiment, or urea which is present in tubular epithelium.

According to a "secretion" theory the secondary return of urea which has been directly absorbed earlier would indicate that the high-level equilibrium between tubular epithelium and blood, reached within a few seconds after the injection, has become disturbed by the fall in the blood urea. This explanation of a period of readjustment is the same as that suggested earlier to account for the lower ratios in long sample experiments that continue about a minute. The question immediately arises—why should a secreting epithelium regain its equilibrium by returning urea to the blood instead of by secreting the urea into the tubular lumen? This objection is not as serious as it appears on first glance. In these experiments the kidney receives a sudden large dose of urea; excretion certainly begins fairly quickly, and probably not many minutes will pass before the tubular urine contains about its maximum percentage of urea. As a result, the secretion of urea is presumably partially inhibited, and the tubular epithelium, overloaded with urea, finds relief by secondary return as well as by what secretion it still remains capable of.

It must of course be understood that the occurrence of a negative renal extraction ratio for urea does not imply that the kidney is not excreting urea at the time. The processes of secretion and secondary return are not mutually exclusive; they may quite well occur simultaneously.

The renal extraction ratios which are low but still positive come into the same theoretical discussion; they include most of the urea experiments and all of the creatinine experiments at the time of the dip in the curves. According to a "filtration" theory, the renal extraction ratio indicates the combined effect of the filtration and of the secondary return of reabsorbed substance; according to a "secretion" theory it indicates

the balance between direct absorption and any secondary return which may occur. The two postulated activities partially counterbalance each other at the "dip" period, and the result is either a low renal extraction ratio or sometimes even a negative one according to whether the balance is a little net filtration or direct absorption or a net secondary return.

Why then is the dip in the urea curve so much longer than that in the creatinine curve? The creatinine in the blood falls more rapidly than urea between 3 and 50 min.; according to a "secretion" theory this should lead to greater disturbance of equilibrium between the creatinine content of the tubular epithelium and that of the blood than in the case of urea. The chief difficulty in answering the question lies in the complete absence of knowledge as to what are the maximum possible percentages of urea and of creatinine in the urine in the different parts of the tubules. It is thus not possible to discuss the relative difficulty in the secretion of the two substances by the tubular epithelium.

General.

If the results are explained on the basis of a pure "filtration" theory, the plasma renal extraction ratios represent the minimum amount of filtration that occurs. Should any secondary return occur, the amount of filtration must of course be higher. The plasma renal extraction ratios for small quantities of creatinine 1 or 2 hours after the injection are about 40 p.c.; those for urea are about 15 p.c. These figures agree quite well with the relative levels of the plasma clearances found by other investigators [Rehberg, 1926; Cope, 1931; Jolliffe and Smith, 1931].

On the view that creatinine is not directly absorbed from the blood the results would indicate that about 40 p.c. of the plasma is filtered off in the glomeruli and that two-thirds of the filtered urea is reabsorbed and secondarily returned to the blood. The lower ratios in many experiments with creatinine require the assumption either that this substance can sometimes be reabsorbed or that the filtration of plasma in these cases was below 40 p.c. The plasma renal extraction ratios for urea during the first minute after its injection are sometimes as high as 50 p.c.; this requires either that some urea is directly absorbed or that the filtration of plasma was as much as 50 p.c.

On the other hand, if direct absorption of urea or creatinine does occur, all the results can be explained by this process alone. If direct absorption be responsible for only part of the elimination of these substances from the blood, some lower amount of filtration than 40 p.c.

may be postulated, and the higher ratios are then a measure of the amount of direct absorption.

From the experimental results it is not possible to adduce any single fact in absolute proof or disproof of either the "filtration" or the "secretion" theory of renal function. A plasma renal extraction ratio of 5 p.c., such as occurs in some of the urea experiments, is certainly low enough to be explained on a simple "filtration" theory. On the other hand, a ratio of 100 p.c., such as is found with certain dyes, is obviously too high to be explained in this way. The ratio must at some level between these two extremes become too high to be accounted for by "filtration" alone; at what point remains an unsolved problem. Although the filtration of half the plasma in the glomeruli as suggested by some of the urea long sample experiments is certainly rather high, it is not in any way impossible on theoretical grounds. Ekehorn [1931] considers it probable that 25–30 p.c. of the blood passing through the glomerular tufts is filtered off; these figures correspond to about half the plasma.

APPENDIX.

Consideration of experimental technique.

Long sample experiment.

The methods of obviating significant errors require brief consideration. The criteria to be given of a satisfactory experiment are necessarily set at arbitrary levels, but they are sufficiently stringent to reduce the possible errors to unimportant proportions. For purposes of clarity, creatinine alone is discussed below. Except where specific differences are pointed out it is to be understood that the discussion applies equally to urea.

Endogenous creatinine and urea.

The renal vein blood contains, in addition to the injected creatinine, a certain amount of endogenous creatinine. This source of error is negligible if the amount of creatinine injected be large; *e.g.* about 100 times the amount normally in the animal's whole blood volume. The actual amount of creatinine injected was always over 100 mg. per kg. of body weight.

An allowance is, however, required for the endogenous creatinine contained in the renal parenchyma and in the urine in the kidney. This amount is relatively not very large because of the diuretic action of the urethane, and also because most of the urine in the renal pelvis escapes when the kidney is taken out of the body and while the perirenal fat

is being removed from it. The necessary correction for the endogenous renal creatinine was found from a series of control experiments on anæsthetized animals which received no injection of creatinine. In order that the correction might be relatively small, the amount of creatinine injected in the long sample experiment was so adjusted that the amount taken up by the kidney was at least three times as much as the average amount in the kidney before experiment. For this purpose the amount injected had to be over 300 times as much as the amount in the kidney before experiment.

It will be seen from Table XVII that the allowance for endogenous creatinine in a kidney at the beginning of a long sample experiment may be taken as 0·4 mg. In order that this should not be of too much importance the amount of injected creatinine taken up by the kidney during the experiment should be over 1·2 mg.; the total amount of creatinine injected should therefore be at least 120 mg.

TABLE XVII. Amounts of creatinine and urea in the kidneys of control rabbits killed half an hour after anæsthetization with intravenous urethane.

	Creatinine (mg.) in	
Rabbit	Left kidney	Right kidney
I 1	0·19	0·17
I 2	Not estimated	0·18
I 3	,,	0·19
I 4	0·22	0·21
I 5	0·26	0·21
I 6	0·24	0·28
I 7	0·56	0·53
I 8	0·65	0·56

Mean creatinine per kidney 0·32 mg.

	Urea (mg.) in	
	Left kidney	Right kidney
J 1	5·9	Not estimated
J 2	7·8	6·9
J 3	8·7	9·8
J 4	9·4	Not estimated
J 5	11·1	10·4

Mean urea per kidney 8·8 mg.

Similarly in the case of urea, as shown in Table XVII, the mean amount of endogenous urea in the kidney is 8·8 mg.; the amount of injected urea taken up by the kidney during the experiment should therefore be over 24 mg.; the total amount to be injected is thus at least 2400 mg.

Injected creatinine and urea.

(1) To ensure that the wave of high creatinine content in the blood had passed through the kidney to the renal vein, the following conditions were observed.

(*a*) The renal vein blood should be collected for more than 25 sec. after the beginning of the injection into the ear vein, and for more than 10 sec. after the end of that injection.

(*b*) At least 5 c.c. of blood should be collected from the renal vein subsequent to 10 sec. after the beginning of the injection.

In the case of urea, in view of the unevenness of the circulation rates, it is more important than ever that the total renal blood flow should be sufficient for the peak of the urea content of the blood to have passed through the kidney to the renal vein. The peak is longer and lower owing to the length of the injection. Therefore, in addition to the ordinary criteria of long sample experiments, a further standard has been set up; the blood collected after the end of the injection of urea must be at least 2·5 c.c.

(*c*) The renal circulation rate should be over 0·75 c.c. per g. per min.

(*d*) The amount of creatinine reaching the operated kidney *via* the renal artery during the experiment should be at least 2·5 p.c. of the amount injected.

(*e*) There should be no evidence that the blood flow to the operated kidney had been affected by any local reflex from the operation; for this, the amount of creatinine taken up by the left kidney, should preferably be within ± 10 p.c. of that taken up by the right kidney. The collection of the renal vein blood must of course be perfectly free and unobstructed.

(2) For the present purpose the urine in the tubules was considered to be in the kidney proper, whereas the blood in the renal capillaries was not. There was some escape of blood and urine from the hilum of the kidney after removal and during the process of cleaning.

(*a*) Hæmoglobin estimations of aqueous kidney extracts showed that the blood remaining in the kidney was very rarely as much as 0·2 c.c. If this blood had the same creatinine content as the renal vein blood, it would raise the amount in the kidney by 1 or 2 p.c. of the observed amount; the effect of this on the renal extraction ratio is negligible.

(*b*) It is unlikely that much of the injected creatinine which had been taken up by the kidney was lost from the hilum; the experiments were not very long, and any injected creatinine in tubular urine was probably not in the collecting tubules in any quantity at the end of the collection

of renal blood. If loss did occur it would lead to under-estimation of the amount of creatinine taken up by the kidney, and thus to under-estimation of the renal extraction ratio.

(3) The long sample experiments can only give average values for the renal extraction ratio during the whole minute that the experiment lasts. It is quite possible that the ratio is high at 10–20 sec. after the beginning of the injection and then becomes smaller. It is also possible that, as the creatinine is rapidly disappearing from the blood, there may be a secondary return of creatinine from the kidney to the blood towards the end of the experiment. Any such secondary return is, however, not to be looked on as an error leading to under-estimation of the net amount of creatinine taken up by the kidney, but as an integral part of this net amount.

Short sample experiment.

The short sample experiment consists essentially of determining small differences of concentration of creatinine between the heart blood and renal vein blood. The differences are so small that very slight errors are of far more serious significance than in the long sample experiments. A few points thus require detailed consideration.

Renal vein blood sample.

(1) Creatinine is a normal constituent of blood and urine. The blood normally contains a small amount of "chromogenic substance" [see Hunter, 1928] which is not creatinine. The actual amount is uncertain, but it is probably large enough in proportion to the normal creatinine content of the blood to introduce gross errors in the determination of the renal extraction ratio in an animal whose blood creatinine is at the normal level.

To render the error due to "chromogenic substances" of no significance, and to permit of accurate estimation of the blood creatinine, it is necessary to raise the blood creatinine artificially, preferably to about twenty times the normal.

This difficulty does not arise in the estimation of the renal extraction ratio for urea at its normal level.

(2) As the differences between the creatinine contents of the heart blood and renal vein blood were very small, the slightest reflex interference with kidney function at the time of the operation might have completely upset the results of any experiment. This matter is very important [see Addis and Shevky, 1917; Nash, 1922]. Much of the older work, and even some more recent, on renal vein blood chemistry

has been vitiated by such reflexes. The only direct information about the question of whether local reflex disturbance of renal function occurs in the operated kidney is obtained from the long sample experiments. As has been shown the agreement between the amounts of creatinine taken up by the two kidneys in those experiments suggests that no such reflex develops. This conclusion may, however, only be transferred to the short sample experiments with the greatest caution. The condition of the kidneys in the two types of experiment is in no way analogous. At the beginning of a long sample experiment the kidney contains only very little creatinine and is suddenly confronted with a large amount in the blood coming to it. Under such a strong stimulus a very gross reflex would presumably be required to inhibit the kidney from dealing with the creatinine. On the other hand, at the beginning of a short sample experiment some minutes after the injection of the creatinine the kidney contains a great deal of creatinine; the amount in the blood is steadily falling. In these short sample experiments the balance between the amount of creatinine in the kidney and that in the blood may be fairly delicately adjusted; if so, a slight reflex disturbance from the operation might upset the balance. The evidence from the long sample experiments cannot therefore be accepted as a definite indication that disturbances of renal function do not occur as a reflex from the operation.

A further point, however, requires consideration. If, owing to the operation, local reflexes develop, they will probably be multiple in type; *i.e.* disturbance of renal function will probably be associated with disturbance of renal circulation, though both may be independent reflexes. Such vasomotor alterations may be expected to be constrictor, from the known facts about the influence of grosser operative interference on the deposition of dyes in the kidney. Thus a slowing down of the renal blood flow during the operation is possibly an indication of reflex disturbance of renal function. A slight slowing down was, however, only seen three times in the present series of experiments and the renal extraction ratios were not abnormal in these cases. Further, the evidence of the long sample experiment shows that the present method of collecting blood from the renal vein does not produce any significant difference between the blood flow to the two kidneys.

It therefore appears highly probable that no special local reflexes occurred in the operated kidney, either vascular or functional. Nevertheless, in order to reduce the possibility of any such reflex developing, the collection of renal vein blood was made as short as possible. Certain criteria of a "satisfactory" experiment were adopted, as in the long

sample experiment. The exact figures are somewhat arbitrary but in practice give a reasonable dividing line between short sample experiments which were really short and those which were unsatisfactorily prolonged; most experiments either fulfilled the required conditions easily, or failed even to approach them.

(*a*) The 3 c.c. of blood required for creatinine estimations must be collected within 30 sec.; the 5 c.c. of blood for urea estimations within 40 sec.

(*b*) The renal circulation rate must be over 0·75 c.c. per g. per min.

(3) Certain of the experimental results raise the question as to whether too forcible aspiration of renal vein blood might lower the pressure in the renal venules so much as to produce pyelo-venous back flow [Hinman and Lee-Brown, 1924]. This does not appear possible. If the suction is too great the wall of the renal vein is drawn down, as a valve on the bevel of the needle point; it is thus impossible to produce a negative pressure in the renal venules. Further, accidental pyelo-venous back flow would lead to widely varying and inexplicable results, whereas the variations in the actual experiments were related to methodical and ascertainable factors. This problem can thus not be considered as of any significance.

Heart blood sample.

The application of a correction to the heart blood sample to allow for the disappearance of creatinine from the heart blood during the experiment is a matter more of theoretical than of practical importance. In the case of dyes where the short sample experiments were performed within a few seconds after the injection this correction was of great significance. In the present experiments it is only in the experiments performed within a few minutes after the injection that it is large enough to require consideration.

It is required to know the creatinine content of that blood which was in the heart about 3 sec. before the mid-point of the collection of the renal vein sample, 3 sec. being approximately the time of circulation of the blood from the heart to the renal vein. The heart blood sample is actually obtained at some time later, usually from 8 to 24 sec. after the mid-point of the collection of the renal vein sample. The amount of the correction depends on two numerical factors: (*a*) the interval elapsing between the collection of the two samples of blood; this was measured in the experiment, and (*b*) the rate of disappearance of the creatinine from the heart blood at the time of the experiment. This rate can be

ascertained by comparing the amounts of creatinine in the heart bloods of a large number of rabbits at known intervals after the intravenous injection of a standard amount of creatinine, and estimating from this the average rate at which the creatinine disappeared from the blood in the whole series of rabbits. This average rate corresponds to what was found by making in a few individual rabbits two measurements of the creatinine content of the blood with an interval of several minutes. The method described above is necessary to determine the rate of disap-

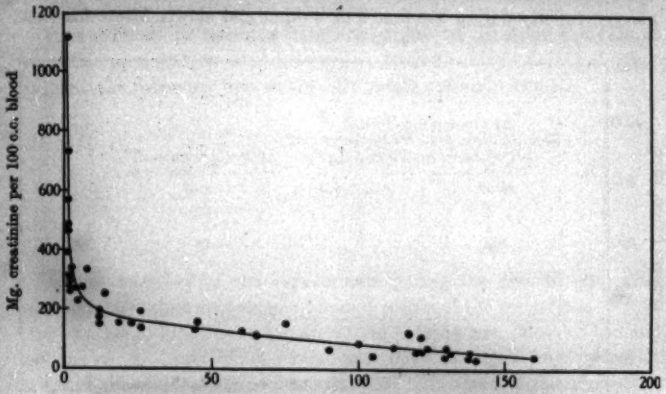

Fig. 8. Rate of disappearance of creatinine from blood.

pearance, as a large series of determinations at very short intervals of the creatinine content of the blood of a single rabbit is unsatisfactory. It was shown [Sheehan, 1931] that this procedure produces a quite abnormal rate of disappearance of substances from the blood.

The rate of disappearance of injected creatinine from the general blood stream is illustrated in Fig. 8. The points indicate the amount of creatinine per 100 c.c. heart blood of individual rabbits operated on at different intervals after the intravenous injection of creatinine. Most of the animals had received only 200–500 mg. of creatinine per kg., but the figures have been calculated to a standard dosage of 1000 mg. per kg. so that Figs. 8 and 9 may be comparable. There is no significant difference between the rates of disappearance of the creatinine from the blood after the small or the large dosage. In only five animals was the creatinine

in the heart blood less than 25 mg. per 100 c.c.; the pre-existent creatinine in the blood is thus relatively so small as to require no allowance in calculations.

An animal which has 100 c.c. of blood weighs about 1·5–2 kg.; on a dosage of 1000 mg. per kg. it would therefore receive 1500–2000 mg. creatinine into its 100 c.c. of blood. The initial level of the creatinine in the blood immediately after the injection would theoretically be about 2000 mg. per 100 c.c. On extending the area of the graph to include this it will be seen that the creatinine disappears from the blood in two chief phases; rapidly during the first 2 or 3 min., and slowly after this time.

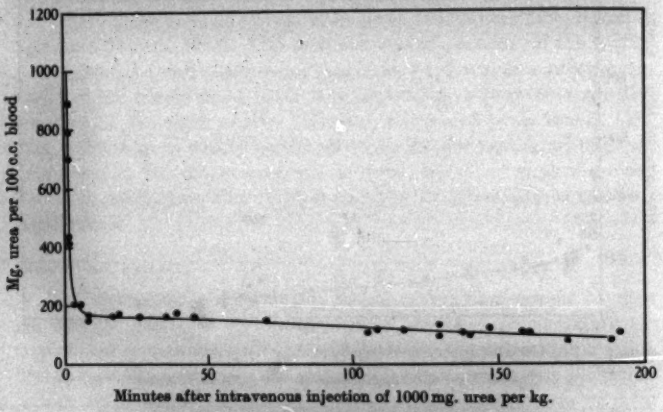

Fig. 9. Rate of disappearance of urea from blood.

In the case of urea various doses were injected. The adjustment of the blood ureas to what they would have been after the injection of a standard dose is interfered with by the need for a correction for urea pre-existent in the blood. This was not measured in the present experiments. In an earlier series of experiments it lay between 16 and 47 mg. per 100 c.c.; this range is too wide to allow of an average figure being adopted, in view of the relatively large size of the correction in cases where only small amounts of urea were injected.

In order to avoid the mathematical multiplication of figures, with the associated multiplication of correction errors, the points given in Fig. 9 are taken only from experiments where 1000 mg. of urea were

injected per kg., except for the figures at 1 min. Here the amount injected was from 1250 to 2500 mg. per kg.; the figures are so high that no correction is required, and the calculation to standard dosage is a division.

The blood urea at the end of the injection is probably about 1500–2000 mg. per 100 c.c. It will be seen that, like creatinine, there is a very rapid fall in the graph during the first 2 or 3 min. (probably due to the passage of urea into various tissues of the body) and subsequently a very slow fall (probably related to excretion).

It is difficult to estimate with any degree of accuracy the rate at which the urea and creatinine disappear from the blood during the first 10 min.; the following figures are only rough approximations.

Time after injection of creatinine or urea min.	Rate of disappearance. Percentage of actual amount in blood disappearing from blood per min.	
	Creatinine	Urea
3	15	5
7	4	1
10	3	0·5

In the majority of the experiments during the first 10 min. after injection, the intervals between the mid-points of the collections of renal vein and heart blood samples were from 6 to 11 sec. Most of the corrections of the heart blood figures are thus calculated from only 1/10 to 1/6 of the above rates of disappearance. The effect of the corrections on the renal extraction ratios is not to raise any of them by more than 1 p.c. except in one single case, rabbit C 1, where the correction raises the renal extraction ratio by 6 p.c. The corrections other than C 1 have been ignored as negligible.

The effect of urea on peritoneal absorption.

In four of the urea experiments an attempt was made to stimulate the renal function by giving an intraperitoneal injection of 50 c.c. of physiologically normal saline about an hour before the operation, but after the intravenous injection of the urea. A rather unexpected result was, however, obtained; the saline was imperfectly absorbed from the peritoneal cavity in the animals with artificially raised blood ureas. The normal rabbit can remove 50 c.c. of saline completely from its peritoneal cavity within a few minutes, but in these animals the peritoneal cavities contained much free fluid half an hour to an hour after the injection of the saline. The free fluid was not accurately measured, but certainly

represented the greater part of the saline injected. The renal extraction ratios were quite normal in all these experiments, and the results have been recorded in the same table as those from animals which received no saline. The only recognizable effect of the intraperitoneal injection was that the operation was slightly impeded, as it was necessary to swab the free fluid out of the left renal pouch before needling the vein.

CHEMICAL METHODS.

Creatinine.

Blood and urine.

The creatinine was estimated by the ordinary method of Folin and Wu [1919]; *i.e.* tungstate protein-precipitations (in the case of blood) and alkaline picrate treatment of the filtrate for colorimetric measurement.

The only points requiring mention are: (1) the picric acid was recrystallized from boiling benzene and answered Benedict's test [1929] quite satisfactorily; (2) the creatinine content of the blood in the present investigations was always high—usually from 25 to 200 mg. per 100 c.c.

The method of estimation gives reasonably constant though rather low results. Estimations of creatinine added to blood in quantities varying from 10 to 200 mg. per 100 c.c. were between 93 and 94 p.c. of the actual at all levels.

Colorimetric readings are very easy at these high levels; an accuracy of ± 1 p.c. is not difficult even with the standard differing widely in strength from the solution under examination. In all the short sample experiments the renal vein blood was read directly against the heart blood as well as both against a standard; the results always showed close agreement.

Kidneys.

The creatinine was estimated in aqueous extracts of the kidneys, made by grinding up the kidney to a thin cream and extracting with about twelve volumes of water for 2 hours in the cold. The suspension was precipitated with tungstic acid in the same way as the diluted blood of the ordinary blood method. On addition of the alkaline picrate to the filtrate there was a sudden development of a greenish tinge, but this was very transient and had no influence whatever on the subsequent colorimetric readings.

Creatinine added to the original kidney paste was estimated subsequently to about 94–95 p.c. of the actual over a range of from 1 to

20 mg. per kidney. When the aqueous extraction was performed with boiling water only about 91 p.c. could be found in the filtrate.

All the figures for the creatinine in kidney and blood given in this paper are thus only about 94 p.c. of the true amounts: no correction has been made for this error, as it is constant throughout and does not effect any of the renal extraction ratios at all.

Urea.

Blood.

The method employed will not be gone into in great detail here, as it is to form the subject of another communication. It is a modification of Marshall's urease method in which the sources of error have been eliminated to a very considerable extent. It estimates only 94–96 p.c. of the absolute amount of urea, but the relative accuracy is quite good. Paired estimations, each on 2 c.c. of blood, were conducted on every blood sample, and in over three-quarters of the cases agreed to within 0·3 mg. per 100 c.c. As the blood ureas in the present work varied from 48 to 326 mg. per 100 c.c., and were usually between 100 and 200 mg. per 100 c.c., this agreement may be considered satisfactory. The results of three short sample experiments have been discarded as the agreement between pairs in one or other sample was unsatisfactory; in these cases the renal extraction ratio calculated from the lower renal vein blood determination and the higher heart blood figure was over 2 p.c. more than the ratio calculated from the higher renal vein figure and the lower heart figure. In the great majority of the experiments recorded, any slight differences in agreement between pairs were insufficient to affect the renal extraction ratio at all.

In the short sample experiments the renal vein and heart bloods were always set up in the same battery of apparatus for the estimation of urea, and the treatment of them was thus identical. Relatively to each other the figures are therefore reliable.

Kidneys.

The urea in the kidneys was estimated by the method of Marshall and Davis [1914]. Each kidney was extracted with 100 c.c. boiling alcohol, 2 c.c. of the solution thus obtained were evaporated to dryness in the reaction tube of the blood urea apparatus, the dry residue was redissolved in buffer phosphate solution in the tube, and the estimation ontinued as for blood urea. The method appears to give almost complete

extraction. As in the case of blood, only about 94–96 p.c. of the urea extracted is estimated in the subsequent chemical analysis, but this error is not of significance as it affects all estimations (both of blood and kidney) equally and so does not influence the renal extraction ratios at all.

SUMMARY.

1. After the intravenous injection of creatinine or urea into rabbits, comparisons have been made of the amounts of these substances reaching the kidney in the arterial blood and leaving it in the venous blood. The experiments were performed at various times, from a few seconds to 3½ hours after the injection, and at varying concentrations of the substances in the blood from about 20 to 200 mg. per 100 c.c. The percentage of the substance taken up by the kidney from the plasma passing through it is termed here the plasma renal extraction ratio.

2. During the first minute after the injection the mean plasma renal extraction ratio for creatinine is 31 p.c.; for urea it is 40 p.c.

3. During the next 20 min. the ratio for creatinine averages 19 p.c.; for urea it averages 2 p.c., and is sometimes negative.

4. Later than 50 min. after the injection the mean ratio for creatinine at low plasma concentrations is 38 p.c.; that for urea at low plasma concentrations is 15 p.c.

5. With increasing concentration of either substance in the plasma, a diminishing proportion is removed by the kidney from the blood. It appears probable that the absolute amount of these substances that the kidney can remove from the blood passing through it is limited by a maximum value of about 20 mg. of creatinine or 15 mg. of urea per 100 c.c. blood.

6. Combined experiments indicate that, when the kidney is removing very large amounts of creatinine from the blood, it removes little or no urea from the blood. When it is removing fairly large amounts of urea from the blood it can, however, still remove its normal amount of creatinine from the blood.

7. To explain all the results by a pure "filtration" theory, it is necessary to postulate the filtration of a third to a half of the plasma in the glomeruli, and the reabsorption of various amounts of urea and creatinine by the tubular epithelium. According to a pure "secretion" theory, the tubular epithelium must absorb various amounts of the creatinine or urea in the plasma directly from the intertubular capillaries; under certain circumstances up to about half. Either theory, or a com-

bination of both, may be correct as no fundamental impossibility is involved in any of the above explanations.

8. Nevertheless, it is definitely established here that under certain conditions a secondary return of urea from the renal parenchyma to the blood does occur.

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THE CONTROL OF THE INSULIN OUTPUT OF THE PANCREAS.

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Although there is abundant evidence that the output of insulin from the pancreas is regulated according to the rate at which sugar enters the blood, yet opinion is divided as to the means by which this regulation is brought about. Some [Corral, 1918; McCormick, Macleod and O'Brien, 1923; Britton, 1925; Ahlgren, 1926; Clark, 1925, 1926, 1927, 1931; Debois, 1930; Zunz and La Barre, 1927, 1928; La Barre, 1928, etc.] regard the pancreas as being under nervous control by the vagus, though they differ among themselves as to whether the vagi (or one of them, and, if so, which one) provoke or inhibit the liberation of insulin, and also as to whether the response is a reflex, or is due to a direct effect of raised blood sugar on the nerve centres.

The alternative would be to consider the stimulus as a hormonal one, the pancreas directly responding to a raised arterial blood sugar by secretion of insulin. There is indeed evidence, *e.g.* that from experiments on denervation or transplantation of the pancreas, etc., which would seem unanswerably to support such a view [Banting and Gairns, 1924; Gayet and Guillaumie, 1927, 1928; Houssay, Lewis and Foglia, 1929; Wulf, 1929; Colwell, 1930, etc.].

An obvious experiment would be that of injecting glucose solution into the pancreatico-duodenal artery, by which the blood sugar would be raised considerably in the vessels of the pancreas but relatively little elsewhere. Such experiments have been carried out, and positive results claimed, by Grafe and Meythaler [1927, 1928], but these have been objected to by Geiger [1927, 1928] on the ground that the solutions of sugar used were grossly hypertonic. Further, both Grafe and Meythaler and Geiger used anæsthetized animals and thus introduced a complication. Accordingly the experiments about to be described were carried out mostly on decapitated cats, thus at once eliminating all possibility of central vagal control and of interference by anæsthetic.

Methods.

Fourteen experiments were completed. The cats were fasted for 20–45 hours, and were then decapitated under ether by Sherrington's method, or (in two experiments) anæsthetized by chloralose. Carotid blood samples were taken at intervals for estimation by the Hagedorn-Jensen method. After the first or second sample the abdomen was opened and the arteries for infusion dissected free; this caused no significant change in the blood-sugar curve, which was spontaneously falling following the decapitational rise. The sugar solution used for infusion was 5 p.c. glucose in saline or tap water, except in one experiment where 40 p.c. glucose in water was used. The slow infusion was made by the use of Burn and Dale's apparatus [1924], either into the pancreatico-duodenal artery, or, for control, into the portal vein or femoral artery. The solution was introduced into the chosen vessel by means of the finest hypodermic injection needle, without any ligation of the vessel; so that the solution mixed with the blood flowing normally and uninterruptedly along the vessel. The greatest care was taken to give uniform and accurately known rates of infusion and, in the later experiments, the rate of flow was regulated by an assistant with a stop-watch who gave his attention solely to this task. At the end of all the later experiments some chicago blue solution was passed in by the needle into the pancreatico-duodenal artery in order to ascertain the area of its distribution. This was found to be the whole head and neck of the pancreas, in all about half the organ. Carotid blood-pressures were observed at intervals, or at beginning and end of the experiments.

Results.

Control injections into the femoral artery appeared to give the same results as those into the portal vein.

As a result of the infusion of the sugar solution into the pancreatic vessels there was, as an immediate result in seven experiments, a rise of general arterial blood sugar, while in the other seven instances a slight fall occurred. In contrast with this, when the control injection was made into the portal vein or femoral artery (with only one exception), a rise of blood sugar always resulted.

Following either infusion there was in all but three instances (two following infusions into the leg, one into the liver) a fall of blood sugar, but the fall which always followed infusion with the pancreas was in

eleven experiments steeper than that following the control infusions; in the remaining three no controls or observations of fall were made.

The following protocols, one in which the infusion into the portal vein preceded that into the pancreas, the other in which infusion into the pancreas preceded the control into the femoral artery, are given by way of illustration.

Exp. 7. 10. iv. 33. Cat (m.), 2·7 kg., fasted 45 hr. Glucose solution, 5 p.c. (in saline), infused into (1) liver through splenic vein, (2) pancreas through pancreatico-duodenal artery. Rectal temperature 36·5° C. throughout.

Min.	Procedure	Blood glucose mg./100 c.c.	Rate of infusion c.c./10 min.	Arterial blood-pressure mm.
0	Ether	—	—	—
25	Decapitation	—	—	—
35	—	243	—	64
50	Operation	240	—	64
95	Infusion into portal vein	209	0	60
100	"	225	3	58
115	Infusion stopped "	243	3	54
125	—	224	—	—
150	Infusion into pancreas	215	0	55
167	"	228	3	—
180	Infusion stopped "	237	3	45
200	—	177	—	45
215	—	155	—	50

Distribution of pancreatico-duodenal supply shown to include head and neck of pancreas.

Exp. 10. 25. iv. 33. Cat (m.), 3 kg., fasted 44 hr. Infusion of glucose solution, 5 p.c. (in saline), made as accurately as possible in rate, by employing a small burette. Both infusions were made in exactly the same way at a rate of 0·025 c.c. per 5 sec., by help of an assistant. Rectal temperature 39·5° C. throughout.

Min.	Procedure	Blood glucose mg./100 c.c.	Rate of infusion c.c./10 min.	Arterial blood-pressure mm.
0	Ether	—	—	—
30	Decapitation	—	—	—
45	—	251	—	60
75	Operation	209	—	61
100	—	177	—	—
135	Infusion into pancreas	159	0	—
145	"	152	3	—
150	Infusion stopped "	166	3	—
160	—	136	—	—
170	—	124	—	—
185	—	113	—	—
195	Infusion into femoral artery	108	0	—
205	"	141	3	—
210	Infusion stopped "	132	3	—
220	—	125	—	—
230	—	110	—	—
245	—	101	—	45

Distribution of pancreatico-duodenal artery to head and neck of pancreas.

Although the blood-sugar level rose during both infusions, its extent in the pancreas infusion was smaller than that of the control, notwithstanding that the former was made for a longer period. The rate of increase during pancreas infusion was 7·3 mg./100 c.c. in 10 min. (22 mg./100 c.c. in 30 min.), whereas that in control infusion was 17·0 mg./100 c.c. in 10 min. (34 mg./100 c.c. in 20 min.).

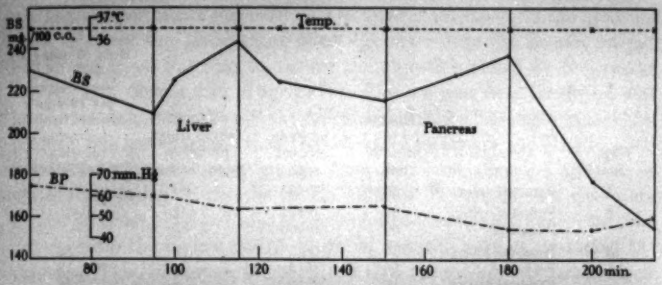

Fig. 1. Effect on blood sugar of decapitated cat of infusion of 5 p.c. glucose (0·3 g. in all) during 20 min. into portal vein and of 0·45 g. during 30 min. into pancreatico-duodenal artery.

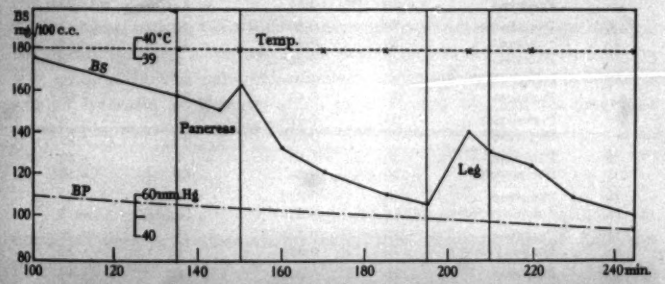

Fig. 2. Effect on blood sugar of decapitated cat of infusion of 5 p.c. glucose (0·225 g.) during 15 min. into pancreatico-duodenal artery and then (0·225 g.) during 15 min. into femoral artery.

On the contrary, the rate of fall in the blood sugar after the pancreas infusion was faster than that in the control, viz. 82 mg./100 c.c. in 35 min., as compared with 28 mg./100 c.c. in 35 min. The results are shown graphically in Fig. 1.

During the pancreas infusion there was, during the first 10 min., a fall of 9 mg./100 c.c., while in the leg infusion there was a rise of 33 mg./100 c.c. In the next 5 min. the blood sugar was increased slightly during the pancreas infusion. But the final rise of the blood sugar in the pancreas infusion was very small as compared with the control.

On the contrary, a more rapid fall in blood sugar was observed after the pancreas infusion than after the control, viz. 53 mg./100 c.c. fall in

TABLE I.

Exp. No.	Infusion into	Duration of infusion min.	Glucose infused g.	Rise in blood sugar produced mg./100 c.c.	Rate of rise (+) or fall (−) in blood sugar per 10 min. immediately following infusion mg./100 c.c.
1	Femoral artery	50	0·445	60	− 9
2	Femoral artery	55	0·415	−101	—
	Pancreas	60	0·450	− 62	—
3	Femoral artery	10	0·15	12	−14
	Pancreas	10	0·15	− 29	−18
	Femoral artery	10	0·10	3	+24
	Pancreas	30	0·325	− 43	—
4	Femoral artery	15	0·15	35	+31
	Pancreas	28	0·42	− 59	−13
5	Portal vein	25	0·375	21	−17
	Pancreas	50	0·60	− 33	−23
6	Portal vein	20	0·30	40	+ 3
	Pancreas	30	0·45	− 12	− 4
	Pancreas	30	0·50	5	− 9
7	Portal vein	20	0·30	34	−19
	Pancreas	30	0·45	22	−30
8	Portal vein	25	0·375	44	− 4
	Pancreas	25	0·375	27	−14
9	Pancreas	30	0·45	− 2	−33
	Portal vein	30	0·45	65	−12
10	Pancreas	15	0·225	7	−30
	Femoral artery	15	0·225	24	− 7
11	Pancreas	15	0·225	36	−38
	Femoral artery	15	0·225	9	−32
12*	Pancreas	15	0·225	6	−14
	Femoral artery	15	0·225	14	− 7
13†	Pancreas	5	0·6	50	−32
	Pancreas	5	0·6	59	−32
	Pancreas	5	0·6	43	−40
14‡	Pancreas	15	0·18	− 21	−14
	Femoral artery	15	0·225	18	0
	Pancreas	15	0·225	12	− 4

* Chloralose.

† Chloralosed animal. Injection of 40 p.c. glucose at rate of 0·3 c.c. per min.

‡ Cat given 0·1 g. thyroideum siccum daily for 16 days previously.

35 min. following the pancreas infusion, and 31 mg./100 c.c. fall in 35 min. following the control infusion. This experiment is shown graphically in Fig. 2.

Table I shows in summary the rates of rise during, and of fall (or rise) following, the various infusions. The last column shows the fall (or rise) in the first 10 min. after termination of the infusion, as observed, or, when no sample was taken at 10 min., as calculated.

These experiments seem not only to answer affirmatively the question as to whether the blood-sugar level directly affects the insulin output of the pancreas, but also to answer the objection raised by Geiger to the work of Grafe and Meythaler. The solution here employed was not hypertonic, and though the fall of blood sugar following pancreatic infusion was less dramatic than that obtained by Grafe and Meythaler, it was nevertheless clearly greater than that seen after the infusion of either liver or leg. Since the results obtained on decapitated cats were similar to those obtained with those under chloralose, it would seem that control from the higher centres plays no essential part in producing the response. This, of course, is not to say that the response is incapable of being modified by nervous action. Moreover, the liver itself appears to play no direct part in causing the effect, because the results of infusion into the portal vein are on the whole similar to those got by infusion into the femoral artery.

One observation, for which no evident explanation suggests itself, is the failure in many instances of the blood sugar to rise when an infusion was made into the pancreatic vessels, although a similar or even a smaller quantity of glucose, when injected into the femoral artery or portal vein, caused a rise of blood-sugar level.

Summary.

Infusion of 5 p.c. glucose into the blood stream along the pancreatico-duodenal artery in decapitated cats, thus exposing about half the pancreatic tissue to a glucose concentration above the normal, is followed by a more rapid decline in arterial blood sugar than occurs when similar injections are made into the femoral artery or portal vein. It is concluded that the pancreas responds promptly to a high sugar content of its arterial blood by a liberation of insulin.

The expenses of the investigation were in part borne out of a grant from the Government Grants Committee of the Royal Society to Prof. C. Lovatt Evans.

I wish to express here my sincerest thanks for this, and to Prof. C. Lovatt Evans for suggesting the work, and for his help and encouragement throughout.

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THE OUTLINE METHOD FOR INVESTIGATING GASTRIC MOTILITY.

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For the satisfactory study of the motility and rate of emptying of the stomach, it seems essential that the method used should involve the minimum of interference with that organ or its contents during the periods of observation. The ideal method should enable records to be taken of the intact stomach in its normal surroundings, and containing nothing but normal physiological contents or specific substances which might be under investigation. The methods in use at the present day possess certain inherent disadvantages as judged by this standard: by overcoming some of these difficulties, it is hoped that the "outline method" described in this paper will provide a useful addition to the technical procedures employed in the study of gastric motility.

The utilization of gastric and duodenal fistulæ has been a recognized procedure ever since the classical case of Alexis St Martin. An animal with a gastric fistula can, however, never be considered to possess a healthy stomach: great care must therefore be taken in the evaluation of results obtained thereby. Carlson [1912] has shown clearly that the hunger contractions demonstrated by Boldireff [1905] in his dogs with fistulæ correspond in type to those observed by Carlson in old or debilitated animals in which the contractions were recorded by means of a balloon in the intact stomach. Clinically it is quite apparent that gastrostomies never satisfactorily replace oral feeding.

The utilization of gastric pouches merits the same criticism. Carlson [1912] investigated the relationship between the movements of a Pavlov pouch and those of the reconstituted stomach; he found the relationship extremely variable and apparently dependent upon the size and condition of the bridge of tissue between the two parts. Robins [1923] could detect no correlation between the movements of a Heidenhain pouch and the parent stomach. Still less certain will be the conclusions

drawn from the movements of a transplanted pouch where all muscular and nervous connections are completely divided [Ivy and Farrell, 1925].

The opaque meal possesses the great advantage of utilizing the intact stomach in its normal surroundings. Its inherent defect lies in the necessity of introducing a mass of foreign material which exerts an unknown influence upon the motor response of the stomach. It forms an admirable comparative method, whereby stomachs can be contrasted in terms of their reaction to a standard opaque meal, and it enables a detailed study of contractions and emptying rates to be made under these conditions. The method has, however, definite limitations, as it does not permit of the study of the empty viscus nor the investigation of the response of the stomach to any particular foodstuff or substance, as the presence of the opaque material may conceivably modify the result obtained.

The fractional test-meal can supply indirect information as to motility: the emptying time for any particular constituent of the meal can be determined, and the secretory curves give a measure of the time taken by the stomach to return to its resting state. It provides, however, no information as to the dimensional changes of the organ during the meal, unless the procedure of periodical withdrawal and return of the gastric contents is adopted: this seems a highly unphysiological procedure, as the well-recognized rapid adaptation of the stomach to variations in the volume of its contents would necessitate a gymnastic display quite foreign to the orderly procedure of the uninterrupted passage of a meal. The tube in the stomach is in itself a drawback owing to the great individual variations in the tolerance of its presence.

The object of the "outline method" is the provision of an animal in which the outline of the stomach can be rendered visible by X-rays without in any way interfering with the contents of the organ. A series of fine silver wire sutures are attached to the serous coat along the optical margins of the stomach; X-ray photographs are then taken, and the line of sutures enables the construction of an outlined diagram of the stomach at any phase of its activity.

The method.

Apart from a few preliminary trials on cats, dogs have been used throughout the work. Some care must be exercised in the selection of the animal: it should be amenable to training, and a preliminary trial in the X-ray room should be carried out. A medium-sized terrier with a broad chest and wide subcostal angle is the most suitable—the latter

point is of importance, as it increases the ease of access to the cardiac region when the sutures are being applied. After trial of several types of material, *e.g.* Michel clips and fine silver clips of various shapes, the most suitable medium was found to be fine silver wire of 0·17 mm. diameter: this can be threaded on fine cleft-palate needles and manipulated with the same ease as stout thread.

The object of the method is to define the outline of the stomach by the application of a series of opaque sutures along the optical margins of the organ. The optical margins of the stomach will vary according to the position employed for photography and with the degree of filling of the organ. The first of these variations can be eliminated by the adoption of a standard position for photography, and we have used a dorso-ventral exposure of the animal standing in its normal erect position. For this standard exposure we have determined the anatomical landmarks which correspond to the optical outline of the stomach, and have applied our sutures accordingly. If the sutures are faithfully applied to the anatomical lesser and greater curvatures, it is found that the suture line accurately depicts the outline of the stomach for the whole of the lesser curvature and the pyloric half of the greater curvature. In the region of the body, however, it is found that the line of sutures falls well within the borders of the fundic shadow, so that in this region the anatomical and optical curvatures do not coincide. Dogs with sutures applied along the anatomical curvatures always showed a marked optical defect in the region of the fundus (Figs. 1, 2), and it was necessary to modify the disposition of the sutures in this region in order to perfect the optical outline. In actual fact, the anatomical greater curvature passes on to the dorsal aspect of the fundus of the stomach, and the optical margin must therefore lie anterior to the anatomical boundary. The following procedure has been adopted for the satisfactory definition of the optical greater curvature: starting at the pylorus, the anatomical greater curvature is followed until the lower pole of the spleen is reached, and from this point the suture line is made to diverge from the anatomical curvature, the sutures being inserted along the anterior surface of the stomach till they reach a point $\frac{3}{4}$ in. internal to the line of reflection of the gastro-splenic ligament at the level of the upper pole of the spleen. At this point the suture line stops, for experience has shown that it is quite unnecessary to attempt to outline the fundic cap, the shadow of which is always clearly visible owing to its contents. It should be noted that the sutures are applied under ether anæsthesia; in this condition the stomach is generally very atonic, and thus gives a wrong impression

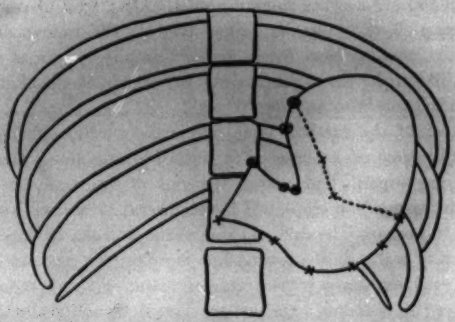

Fig. 1. Fasting stomach of Dog G 5. ⊗ Sutures on lesser curvature. × Sutures on greater curvature. In this dog, the sutures had been placed along the anatomical curvature; hence the marked defect in the outline of the fundus as shown by the dotted line. The continuous outline of the fundus was obtained from the shadow of the fundic contents.

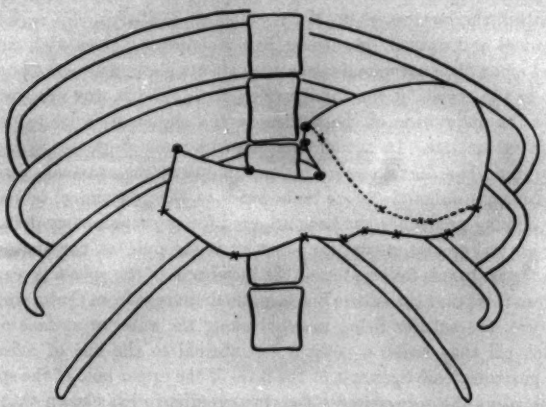

Fig. 2. Fasting stomach of Dog G 6. Tubular stomach suggesting poor tone. ⊗ Sutures on lesser curvature. × Sutures on greater curvature. These sutures were applied to the anatomical curvature giving similar defect as Fig. 1.

as to the width of the gap between the end of the suture line and the cardia. When the animal recovers and the fasting stomach is X-rayed, one is surprised to find how closely the suture line approaches to the cardia (Fig. 6).

Technique.

Under ether anæsthesia, the abdomen is opened through a left paramedian incision, and the stomach is picked up in sponge rubber forceps which allow of the necessary manipulations with the minimum of trauma. Starting at the cardia, a series of fine silver sutures is applied along the lesser curvature at the point of attachment of the anterior layer of the lesser omentum. When the pylorus is reached, attention is directed to the greater curvature, and the sutures are applied along the optical outline as previously described. The sutures are applied under direct observation, and great care is exercised to avoid vessels and nerves. Each suture just penetrates the serous coat, and is tied in a loose loop to avoid strangulation of the included tissue. The number of sutures employed depends upon the size of the stomach and the particular object of the preparation; for the general purpose of determining the whole gastric outline, the sutures are applied at intervals of $\frac{1}{2}$ in., so that about eight are required for the lesser curvature, and about fifteen for the greater. The stomach is then inspected for bleeding points and loose or projecting pieces of wire, the abdomen is closed in layers, and a bandage applied.

After the operation, the animal is returned to a cage in a warm room, and morphia injected to keep it quiet for the ensuing 24 hours. A fluid diet is given for the following 2 days, and the animal then returns to its own cage and a normal diet. Adopting this procedure, we have had no trouble with the healing of wounds, and the animal's appetite after the first days seems quite unaffected by the manipulations.

The subsequent history of animals so treated suggests that they suffer no disability or discomfort from the presence of the sutures. Their appetites, weight and general activities are unimpaired. Of the six animals in which this method has been used, two are alive and well after intervals of 15 and 12 months respectively. Dog G 5 was killed at the end of 18 months, Dog G 6 at the end of 23 months, and Dog G 8 at the end of 4 months, in order to investigate the condition of the stomach. Dog G 7 died of suppurative hepatitis 2 months after the operation. Post-mortem examination of these stomachs showed the sutures in position with no evidence of any local damage to the muscle or mucosa:

there were little patches of peritoneal thickening round each piece of metal, but no adhesions or deformity of the stomach could be seen. From these observations it seems justifiable to conclude that the presence of these sutures in the serous coat produces no significant effect upon the stomach.

Method of recording.

In order that the photographic records shall be strictly comparable, it is essential to establish standard conditions of exposure, and for each animal there must be a fixed relationship between the position of the tube, animal and film. To ensure this uniformity of conditions, the animals

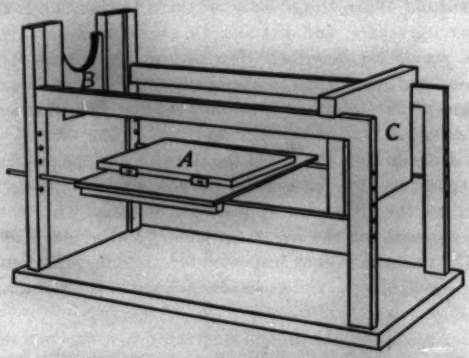

Fig. 3. X-ray stand for dog. A. Film cassette on adjustable platform. B. Adjustable neck-piece. C. Moveable tail-piece.

are taught to stand in a wooden frame which ensures an erect attitude without undue restriction: a centring device correlates the position of the tube and film relative to a fixed point on the animal's spine.

The frame consists of a firm rectangular wooden base with wooden uprights at each corner. The two front uprights are united by a slatted board with a curved neck-piece which can be adjusted to the height of the dog. On each side the uprights are connected by top-rails which prevent the animal lolling to one side, but allow it to stand freely in the erect attitude. The back of the frame is left empty to allow the animal to enter; when the animal is in the frame, with its head projecting over the neck-piece, a tail-piece is adjusted behind so that the animal is enclosed on all sides (Fig. 3). The frame is essential to ensure a uniform

posture, but its dimensions are such that it offers no restriction provided the animal stands erect.

When the animal is in position, a wooden platform carrying the film cassette is slid into position under the dog's abdomen—the height of this platform is adjustable, its setting being fixed for each dog to ensure uniform relationship between the animal and film. The tube is set at a standard height of 24 in. above the film, and by means of a plumb-bob it is centred over the dog and film. To aid this centring, a lead bullet is fixed with collodion in the middle-line of the dog's back over the first lumbar vertebra, and the film cassette has two pairs of cross-wires which, by their intersection, outline a square centimetre over the centre of the film. With very little practice, the dog can be taught to stand in the frame, and the plumb-bob of the tube is aligned with the lead shot and wires in the transverse and longitudinal axis of the dog. The exposure is therefore made under strictly standard conditions. The X-ray film shows the position of the cross-wires and lead shot, and if centring has been satisfactory, the shot should lie in the centre of the square over the first lumbar vertebra.

Using a 30 M.A. fine focus Coolidge tube, 98 K.V., 60 M.A., exposures of 1/20–1/10 sec. were found sufficient.

With this technique it is possible to make photographic studies of the empty stomach and to record the changes in outline during the passage of a meal. Tracings are taken from the films, and the gastric outline is constructed from the line of sutures and the fundic shadow.

In order to obtain some simple quantitative measurement which would serve as an index of the size of the gastric outline, measurements have been made of the length of the lesser and greater curvatures. The length of the lesser curvature shows very little alteration for any phase of gastric filling, and cannot be correlated with changes occurring in the dimensions of the stomach (Fig. 4). The greater curvature, however, shows very definite alterations in length, and this measurement can be utilized for studying the rate of emptying of the organ. If the length of the greater curvature is plotted against the time intervals following the ingestion of a meal, a curve is obtained which shows a steady diminution in the length of the curvature until a stationary level is reached—the time at which the curve flattens out corresponds with the time of emptying of the stomach (Fig. 5). This method of measurement has been employed in all the experiments on the rate of emptying of meals, the curves providing a simple and rapid method of comparison.

It might be objected that the measurement of one selected portion

of the gastric outline provides an unfair index of the dimensional changes of the organ. It must be clearly understood that no attempt is made to establish any quantitative relationship between the length of the greater curvature at any moment and the corresponding gastric volume beyond

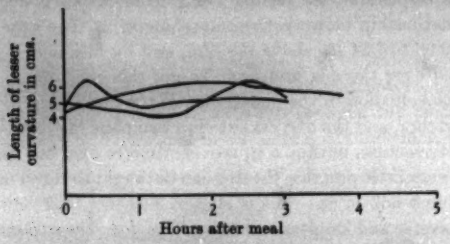

Fig. 4. Length of lesser curvature after 350 c.c. milk. Three similar meals on G 5. Note the uniformity in length at all stages after meal.

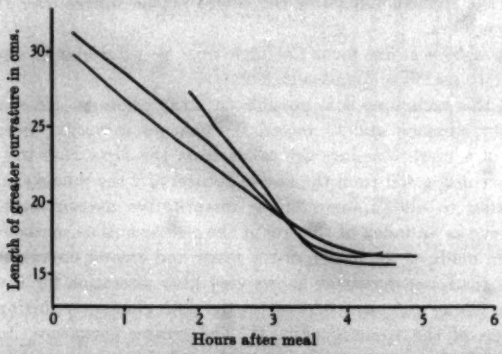

Fig. 5. Length of greater curvature after 350 c.c. milk plus 1 g. NaCl. Three similar meals on G 5. Note the steady diminution in length till the curves suddenly flatten out at the emptying point of 3½–4 hours.

the assumption that, in the same animal, similar values of the length of the greater curvature indicate approximately similar gastric dimensions. Inasmuch as the stomach is a muscular tube, the variations in the capacity of which are made mainly at the expense of the greater curvature, it would appear quite reasonable to use the latter measurement as an index of changes occurring in the former.

The fasting stomach.

Studies have been made of the gastric outlines of dogs which have been deprived of solid and fluid food for 24 hours. This procedure is a routine preliminary to all the experiments we have made. For the stomach under such conditions the usual term "empty" is a misnomer, as some gastric contents are invariably present: consequently, we have adopted the term "fasting" as being more accurately descriptive.

The two dogs employed in the earlier observations by this method present very different forms of gastric outline. Dog G 5, a lively wire-haired terrier, has a definitely J-shaped stomach (Fig. 1), whereas the stomach of Dog G 6, a placid, lethargic Irish terrier, appears as a tubular structure slung transversely across the abdomen (Fig. 2). As these photographs are all dorso-ventral exposures, the presence of a definite J-shape indicates good muscle tone, whereas the simple transverse tubular organ suggests a hypotonic viscus. These differences can be paralleled in the case of the human stomach, bearing in mind the difference in attitude between biped and quadruped. The long dependent J-shaped stomach, typical of hypotonia in man, corresponds with the transverse tube of Dog G 6, whilst the "steer-horn" stomach corresponds to the J-shaped stomach of Dog G 5. The general outline shows clearly that the fasting stomach is not a sac in which the contents collect in the most dependent parts: it is a tube with walls in sufficient tone to maintain the resting contents in the fundic portion, and closely corresponds with the form described by Stopford [1913] for the human stomach.

Waves of contraction.

It has been possible to demonstrate the presence of waves of contraction in the fasting stomach, and with experience a series of exposures at 15–30 sec. intervals can be made with the animal remaining motionless in the frame. A comparison of the outlines of such a series of photographs demonstrates the presence of waves of contraction, and an approximate estimate of their periodicity and duration is possible. Attempts to modify the activity of the empty stomach by disturbing sounds or savoury smells produced no definite results.

Influence of external temperature upon gastric tone.

After numerous observations had been made on these dogs under uniform conditions, the dimensions of the outline of the fasting stomach were well established and were remarkably constant for each animal.

Quite suddenly it was noticed that there was a very definite increase in the size of the fasting stomach: this was so definite that the animal attendant was accused of feeding the animals during the period when they should have been deprived of food. In a search for the cause of this sudden alteration, it was noted that the change coincided with the installation of thermostatically controlled electric radiators in the animal house. Previously the rooms were heated by hot water pipes, and during the winter the animals were subjected to considerable fluctuations between day and night temperature. As soon as the heating was stabilized, the animals became more peaceful, and at the same time the dimensions of their fasting stomachs increased. In order to test the connection between the two phenomena, the electric heating was discontinued, and it was found that when the minimum nocturnal temperature fell to about 45° F., the original small stomach was obtained. The change to the uniform electrical heating produced a 17 p.c. increase in the length of the greater curvature of Dog G 5, and an increase of 23 p.c. in Dog G 6. This variation in the dimensions of the fasting stomach suggests an alteration of gastric tone as part of the reaction of the animal to fluctuations in external temperature.

The passage of a meal through the stomach.

As a basis for comparing the rate of emptying of various foodstuffs, a meal of skim milk has been used as a standard, and the emptying time determined for each dog. The volume of the meal is adjusted to the size of the animal—small dogs receiving 250–350 c.c., large dogs, 500 c.c. The milk is given slightly warmed, and the animal drinks it readily. Skim milk has been chosen as being practically fat-free, isotonic with blood and readily taken by the animals. The routine procedure adopted is as follows. The animal is deprived of food for 24 hours, a photograph of the fasting stomach is taken, and then the meal is given. A photograph of the full stomach is taken and repeated at intervals of an hour until the stomach approaches its fasting dimensions, when the exposures are made at intervals of half an hour. When two consecutive photographs show that the stomach has reached its stationary fasting level, the meal has left the stomach.

A comparison of the outline and distribution of sutures in the fasting and full stomach shows very clearly that the main increase in gastric capacity is made at the expense of the greater curvature of the stomach, particularly in the fundus and proximal part of the body. In the fasting

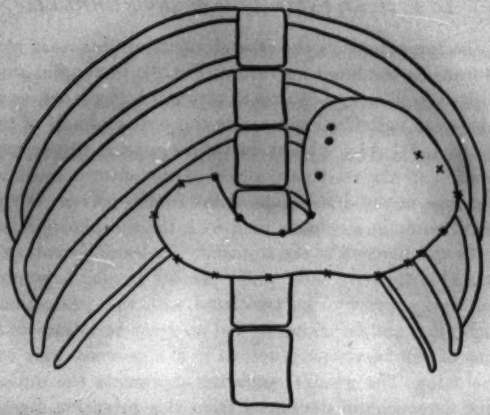

Fig. 6. Fasting stomach of Dog G 9. Eight sutures on lesser curvature, thirteen sutures on greater curvature. These sutures were applied to the "optical" curvatures (see text) with consequent improvement in outline.

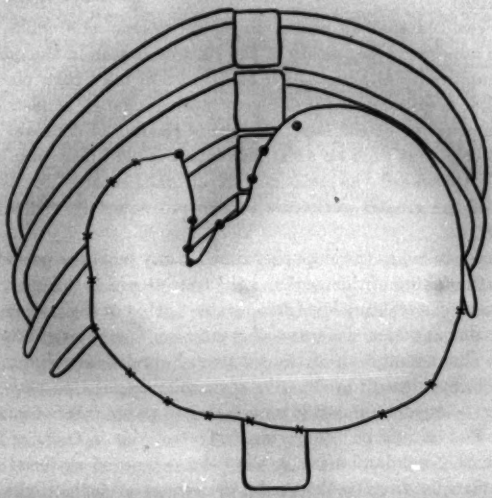

Fig. 7. Stomach of Dog G 9 1 hour after milk meal. Eight sutures on lesser curvature, thirteen sutures on greater curvature. Note the increase in interval between the cardiac sutures of each curvature.

stomach the top suture on the greater curvature lies very close to the cardiac suture on the lesser curvature (Fig. 6): in the full stomach the interval between these two sutures is very much increased, as is also the interval between individual sutures on the proximal half of the greater curvature (Fig. 7). As the lesser curvature appears to vary very little, this alteration in the relative position of the sutures must indicate a marked change in the dimensions of the fundic portion of the greater curvature. This finding is in keeping with the morphological difference between the two portions of the stomach. The lesser curvature is related to the primitive digestive tube, which persists as such in ruminants and kangaroos, and appears to be associated with the conduction of fluid. Accordingly, it is not surprising to find no great variations in the linear dimensions of the lesser curvature, as it is concerned with conduction and not storage. The greater curvature represents the outline of the cæcum-like diverticulum developed from this primitive digestive tube for the purpose of accommodating food; as such, it must be capable of adaptation to alterations in the volume of its contents and, according to the degree of filling, variations in the dimensions of its outline are to be expected.

It is realized that an alteration of the gastric axis could, in itself, alter the apparent relationship of the sutures as seen in the one plane of vision employed. Although such an alteration may take place, we do not think it is responsible for any very marked change in gastric outline, as the dimensions of the lesser curvature remain so constant and it is very unlikely that such an axial movement could affect one curvature only. Furthermore, the relationship of the sutures to the actual shadow of the greater curvature (which is frequently visible) remains constant.

We have taken as the emptying time for any meal the period elapsing between the taking of the meal and the time at which the gastric dimensions assume a stationary level comparable to that of the fasting stomach, *i.e.* the point at which the curve of greater curvature length flattens out (Fig. 5). This assumption of the stationary condition suggests that the stomach has returned to its resting state following the passage of all the food into the duodenum, but it is necessary to prove this before accepting the above as criteria of "emptying." To test this, a trace of barium is added to the standard meal so that the escape of material from the stomach can be directly observed—the record of such "light" barium meals shows that the point of emptying of the meal does coincide with the assumption of a stationary level of gastric dimensions: therefore we

feel justified in adopting the above criteria as a basis for the comparison of "emptying times."

Adopting this standard of measurement, repeated observations have been made on the dogs; the emptying times for each animal have been remarkably constant, though wide variations occur between individual animals.

TABLE I.

Dog	Meal	Emptying time hrs.
G 5	350 c.c. skim milk	3
		3½
		3½
		3
G 9	250 c.c. skim milk	3½
		3½
	350 c.c. skim milk	4½
		4½
G 6	500 c.c. skim milk	4½
		4½
		5

In ordinary X-ray pictures one frequently sees shadows which might possibly indicate the outline of the stomach, but, owing to the uncertainty of their identification, very little reliance can be placed upon them. The presence of the suture-line immediately picks out those shadows related to the stomach, so that its outline can generally be mapped out with an accuracy not directly dependent upon the actual sutures—this fact serves as a very useful counter-check upon the position of the sutures, and with a little practice one soon learns the degree of accuracy with which the sutures have been placed in the individual dogs.

DISCUSSION.

The "outline method" for gastric investigation possesses certain definite advantages over the other methods available. It provides an animal in which the dimensions of the stomach can easily be followed without in any way disturbing that organ or its contents. It is therefore well adapted to the study of the emptying rate of the stomach for various foodstuffs or to the study of the influence of specific substances upon this rate. It enables one to assess the part played by the various portions of the stomach in accommodating the gastric contents and can demonstrate simultaneously segments which are filled or empty. It appears to be the only method available for studying the "fasting stomach" in its normal surroundings.

In use, it has proved to be a practicable method. Animals of this kind have been employed for over two years upon studies of the influence of osmotic pressure and fat content of meals. The presence of the sutures appears to exert no deleterious influence for periods of at least two years. With a little practice the photographs are easy to interpret, and the outlines can be rapidly drawn and measured.

The method appears capable of development in two directions. By a closer distribution of the sutures in any one segment of the stomach, a more detailed analysis of its activity should be possible: this is particularly true of the distal half of the stomach where a strictly accurate outline is most easily obtained. It should also be applicable to other hollow viscera, such as the bladder, gall bladder, uterus and parts of the colon where axial rotation is not prone to occur.

Summary.

A method for studying the motility of the stomach is described. The method consists of applying interrupted fine silver wire sutures to the optical margins of the stomach. X-ray photographs of the gastric outline are taken at any desired phase of gastric activity.

Variations in size and waves of contraction of the fasting stomach have been studied in different animals. The size has been found to be affected by external temperature.

Dimensional changes of the stomach during the passage of a standard milk meal are described.

One of us, W. R. Spurrell, is indebted to the Medical Research Council for a personal grant.

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INFLUENCE OF OSMOTIC PRESSURE UPON THE EMPTYING TIME OF THE STOMACH.

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The influence exerted by the osmotic pressure of the gastric contents upon the rate of emptying of the stomach has been investigated by numerous workers, and while all observers are in agreement that hypertonic solutions lead to delay in the emptying of the organ, considerable difference of opinion exists as to the effects of hypotonic solutions. These results have been obtained by the use of two types of experimental method: (*a*) the determination of the volume of the meal remaining in the stomach after an interval, (*b*) the measurement of the quantity expelled through a duodenal fistula.

Best and Cohnheim [1910] used dogs with a gastric fistula, and measured the volumes of gastric contents 10 minutes after the introduction of meals of various osmotic pressures. Macleod and others [1930], using rats, killed the animals with a blow on the head, rapidly isolated the stomach and intestine, and determined the proportion of meal present in those segments. These methods can give no data as to the total emptying time, and involve considerable interference with the normal physiological condition of the stomach. Powerful contractions of the stomach are frequently seen as an animal dies, and post-mortem findings as to the relative volume of gastric contents may be very misleading as a result of a few terminal gastric convulsions.

Carnot and Chassevant [1905] and Otto [1905] measured the outflow through duodenal fistulæ: they obtained measurements of the total emptying time and studied the changes in the osmotic pressure of the escaping fluid. The modern view of the part played by duodenal absorption in regulating gastric motility introduces an element of uncertainty into results based on such fistula methods. The position of the fistula is usually just below the point of entry of the bile duct, so that the area available for duodenal absorption is very small and the contents are rapidly drained away through the fistula. It may be that the rate of

emptying under these conditions is mainly controlled by the conditions existing in the stomach itself with little or no modification from the duodenum.

Our attention was drawn to this question during an investigation of the properties of certain commercial "meat extracts" which enjoy a reputation as "gastric stimulants." In acute experiments upon the movements of the stomach, the use of these extracts had become a routine to ensure an active preparation. After establishing the emptying time for standard milk meals in dogs by the "outline method," commercial "meat extract" was added to the milk with the expectation that it would accelerate the emptying of the stomach, but on the contrary the meal produced marked delay. In a search for the explanation of this delay, our attention was drawn to the high salt content of these extracts, and experiments were performed to see whether the alteration in osmotic pressure resulting from the addition of the extract to the milk was in any way a contributing factor. The "outline method" [McSwiney and Spurrell, 1933] seemed very suitable for such investigations, as no disturbance of the normal relations of the stomach and duodenum is incurred.

Method.

Hypertonic solutions. The addition of commercial "meat extract" in the quantities employed altered the Δ of the milk meal from 0·55° C. to 0·91° C. Meals of the same osmotic pressure were made up by the addition of NaCl or glucose to the milk. The addition of 2 g. NaCl or 11 g. glucose to 350 c.c. milk raised the Δ of the mixture to 0·9° C., and these meals were given to two adult dogs prepared for the "outline method." Meals with an intermediate osmotic pressure were also prepared by the addition of 1 g. NaCl or 5·5 g. glucose to 350 c.c. meal. All meals were duplicated in each dog. Table I gives a typical series of results for Dog G 5 and Fig. 1 gives a graphic record of the corresponding alterations in the length of the greater curvature.

Table I. Emptying time for meals of different osmotic pressure.

Dog G 5. Meal = 350 c.c.

Meal	Depression freezing point of meal ° C.	Emptying time hrs.
Milk	0·55	3–3½
Milk + glucose	0·712	4½
Milk + NaCl	0·723	4–4½
Milk + NaCl	0·905	4½
Milk + meat extract	0·913	5
Milk + glucose	0·925	4½

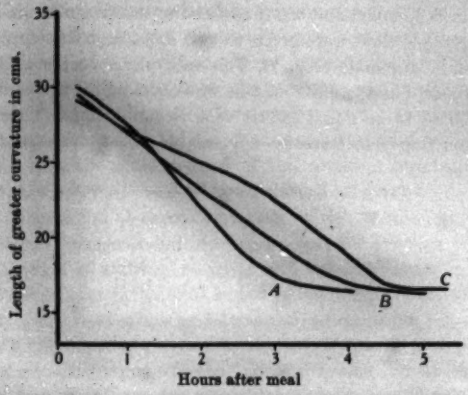

Fig. 1. Lengths of greater curvature after hypertonic meals: A. 350 c.c. of skim milk Δ 0·55° C. B. 350 c.c. of skim milk + glucose Δ 0·712° C. C. 350 c.c. of skim milk + glucose Δ 0·925° C.

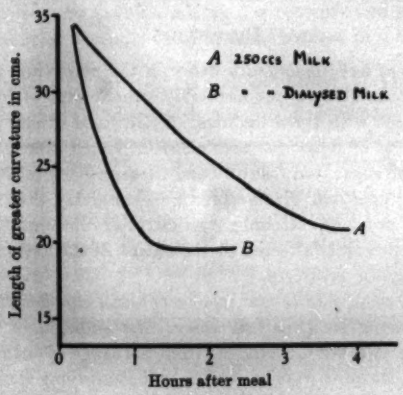

Fig. 2. Lengths of greater curvature after hypotonic meals: A. 250 c.c. of skim milk Δ 0·55° C. B. 250 c.c. of dialysed milk Δ 0·035° C.

Hypotonic solutions. Milk was dialysed against three changes of distilled water in 24 hours and the Δ reduced to 0·035° C. Meals of 250 c.c. of this hypotonic fluid were given to two dogs, and the emptying time determined in duplicate (Fig. 2). The milk underwent a little dilution during dialysis, but the effect of this was checked by the control meals of milk diluted to an equal extent with isotonic saline. This degree of dilution was found to have no appreciable effect upon the emptying time.

TABLE II. Emptying times for hypotonic meals.

Meal = 250 c.c.

Dog	Skim milk hrs.	Dialysed milk hrs.
G 9	3½–3¾	1½–1¾
G 10	3¼	1¾–2¼

Experimental results. Hypertonic solutions produce delay in emptying proportional to the rise of osmotic pressure employed. As iso-osmotic mixtures of milk and commercial "meat extract," milk and NaCl, milk and glucose produce a similar degree of delay, it is clear that the delay is not due to any particular constituent of the meals but to the common factor of hypertonicity. Hypotonic solutions (as seen from Fig. 2) empty more rapidly than isotonic.

DISCUSSION.

All observers have recorded a delay in the emptying of hypertonic solutions, and the delay is proportional to the degree of hypertonicity: our results agree with these findings. Miller and others [1920] found the same degree of delay for test meals of hypertonic glucose as for hypertonic cane sugar, but Carnot and Chassevant [1905] considered that meals of hypertonic glucose produced less delay than saline of the same osmotic pressure. According to our observations, the degree of delay is not appreciably affected by the nature of the material employed to raise the osmotic pressures.

It is rather surprising to find relatively small changes in the osmotic pressure producing such marked delay. The addition of 2 g. NaCl to 350 c.c. skim milk is not far removed from the range of ordinary culinary procedure, so that quite wide variations in the emptying time of standard meals may be expected if strict attention is not paid to the salt content.

One is faced with considerable difficulty in assessing what is generally meant by the emptying time for a meal. If the meal contains one single constituent, the time of its disappearance from the stomach is obviously

the emptying time. Meals rarely consist of a single constituent, and it is not justifiable to select a particular component, follow its course through the stomach and apply the results obtained to the whole meal, *e.g.* the emptying point of a gruel meal is not necessarily the point of disappearance of carbohydrate from the stomach. It would appear more reliable to determine the time taken for the stomach to resume its resting state, *i.e.* to determine the period of gastric activity in response to a meal, and it is the measurement of this quantity that we have made and compared by the "outline method."

The dimensional changes undergone by the stomach as the result of a meal would appear to depend upon a balance between three factors:

(1) Amount of material escaping into the duodenum.

(2) The volume of material added to the gastric contents, *i.e.* gastric secretion, swallowed saliva and duodenal regurgitation.

(3) The absorption of water by the stomach.

All observations on the rate of pyloric outflow have shown that this is diminished in the case of hypertonic solutions. Carnot and Chassevant [1905], Macleod and others [1930] found evidence of duodenal irritation as a result of the escape of such solutions, and it seems probable in their experiments that the pyloric mechanism was affected thereby. In addition, the presence of hypertonic fluids in the stomach gives rise to a copious secretion which dilutes the contents and greatly increases their volume, *e.g.* a 60 c.c. meal of meat extract in a cat will produce 40 c.c. of secretion in an hour, and 20 p.c. glucose in the rat's stomach is diluted 40–90 p.c. in 1 hour [Macleod and others, 1930]. The amount of water absorbed by the gastric mucosa is influenced by the osmotic pressure of the gastric contents, and hypertonic solutions will tend to diminish this process. Thus, all three factors will unite in maintaining the volume of the gastric contents and so producing a delay in emptying.

In respect of hypotonic solutions, Carnot and Chassevant [1905] state that they escape through the pylorus slightly more slowly than isotonic, though they give no details of measurement. Otto [1905] also makes the same statement, but graphs constructed from his protocols give exactly the same emptying time for iso- and hypotonic solutions. Best and Cohnheim [1910] found that the residual volume of gastric contents 10 min. after a meal varied very little for iso- and hypotonic solutions.

In rats, Macleod and others [1930] found that, following the ingestion of hypotonic glucose solutions, the volume of the gastric contents rapidly diminished, although the glucose concentration remained almost

unaltered. This finding points to a rapid rate of pyloric outflow with little or no dilution of the gastric contents by secretion. Our experiments on dogs also show a rapid diminution in the volume of the gastric contents. It is probable that the failure of Carnot and Chassevant [1905] and Otto [1905] to demonstrate more rapid pyloric escape with hypotonic solutions may be associated with the use of duodenal fistulæ: in all experiments where the intact gastro-intestinal tract was employed, rapid emptying of hypotonic solutions was recorded. If absorption of water plays any part in the shrinkage in volume of the gastric contents, its effect should be more marked in the case of hypotonic solutions.

From the above considerations, it appears probable that the rapid resumption of the resting dimensions of the stomach following a hypotonic meal depends upon a rapid rate of pyloric outflow, little or no secretion of a diluting fluid and the tendency for an increase in the absorption of water by the gastric mucosa.

Summary.

1. The emptying time of the stomach for hypertonic and hypotonic meals has been determined by the "outline method."

2. Hypertonic meals produce delay in proportion to the degree of hypertonicity.

3. Hypotonic meals leave the stomach more rapidly than isotonic meals.

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BLOOD LACTIC ACID IN MAN DURING REST.

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The problems connected with lactic acid production in the animal body have been extensively investigated by previous authors, but the obvious difficulties encountered in working with the intact organism have rendered the solution to some of these still a matter of speculation. When, for instance, the organism is subjected to a severe stress, the presence of lactic acid in the circulating blood can be readily understood, but its constant presence in small concentration, even during complete bodily rest, is less easy of interpretation. No satisfactory explanation has, in fact, yet been given either for the origin of blood lactic acid in the resting state or for the wide variations in its concentration. The existence of some muscular activity during bodily rest has led most authors to attribute the blood lactic acid to this source. In this paper, however, an attempt will be made to show that muscular activity can have little influence in this connection and that conversion of blood sugar (glycolysis) is the most probable source of this lactic acid. The wide variations in the resting levels of blood lactic acid will then be explained as one of the factors through which the organism endeavours to maintain a constant blood *p*H.

Experimental.

In some of the experiments blood was taken from one of the superficial veins of the arm after initial compression, either manual or by tourniquet, which was released as soon as the needle was in the vein. The subjects were normal young adult males. In other experiments the femoral artery, femoral vein and jugular bulb were punctured, no compression being used. The subjects of these experiments are described in the text. The blood was collected into a small amount (approx. 0·03 g. per 15 c.c.

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blood) of Evans's 9 : 1 mixture of potassium oxalate and sodium fluoride, part of which was placed in the receiver and a smaller part on the barrel of the syringe. After thorough mixing the specimens were placed in a refrigerator or a portable ice-box.

Deproteinization was carried out by the Folin-Wu tungstate-sulphuric acid method. Actually 2 c.c. of whole blood were pipetted into a large centrifuge tube containing 14 c.c. of distilled water and 2 c.c. of 2/3*N* sulphuric acid; 2 c.c. of sodium tungstate were then added and the contents well shaken before being centrifuged. The customary method is then to remove sugar by treatment with copper sulphate and lime. It was found, however, that this was an unnecessary procedure, since by the Friedemann method of lactic acid determination the amount of bisulphite binding material formed from the small amount of glucose present is negligible. On the other hand, when trichloroacetic acid was used as deproteinizing agent, it was usually necessary to treat the filtrate with copper sulphate and lime, not, presumably, to remove sugar, but to remove bisulphite-binding material other than lactic acid which remained in solution or suspension. The results recorded in Table I illustrate these points.

TABLE I.

Deproteinizing agent	Material	Lactic acid in mg./100 c.c. of whole blood: Filtrate treated with copper sulphate and lime	Filtrate used directly
Sodium tungstate and sulphuric acid	Sheep's blood	27·9	27·0
"	" "	29·2	29·2
"	Human blood	12·6	12·6
"	" "	14·4	14·9
"	" "	17·7	17·7
"	Human blood after exercise	54·9	55·8
Trichloroacetic acid	Human blood	12·6	15·5
"	" "	18·7	23·4
"	Human blood after exercise	54·4	59·1

Lactic acid was determined in 10 c.c. of the filtrate by the method of Friedemann, Cotonio and Shaffer, modified by Friedemann and Kendall [1929]. The apparatus used was that designed by Davenport and Cotonio [1927], except that the inlet tube of the boiling flask was connected by means of rubber tubing to the air outside the building. In these circumstances the "blank" value obtained was constant and amounted to 0·15 c.c. of 0·002*N* iodine solution. In all experiments at least two estimations were performed, the specimens being separated before deproteinization. In the great majority of cases, duplicate results agreed to within 0·2 c.c. of 0·002*N* iodine solution, *i.e.* approximately

2 mg./100 c.c. of lactic acid. Any experiment in which duplicate results showed a difference in excess of this figure was ignored. Similarly, in comparing the lactic acid levels of blood taken under varying conditions, differences below 2 mg./100 c.c. were regarded as insignificant. The percentage recovery of added lactic acid (as zinc lactate) was between 95 and 98.

On some of the blood samples *p*H measurements were made with the glass electrode apparatus designed by Kerridge, the precise technique employed being that previously described by one of us [Hurst, 1932]. For this purpose the blood was collected under paraffin.

Blood lactic acid at rest.

The introduction of the Clausen method in 1922 gave a fresh impetus to work on this subject, since previously no thoroughly reliable and convenient technique had been known. Using this method or one of its more recent modifications, numerous investigators have found that the resting levels of the lactic acid of whole blood of man vary within wide limits. Clausen [1922] gave results varying from 15 to 32 mg./100 c.c., whilst Owles [1930], with more refined technique, obtained results varying from 7 to 18 mg./100 c.c. The results of authors who used other methods for lactic acid determination are in close agreement and it seems clear that the normal resting levels differ by as much as 100 p.c.

In the present investigation 26 estimations on 9 normal subjects showed variations between 8·4 and 16·6 mg./100 c.c. (Table II). In each experiment the subject was in the post-absorptive state and was either in bed or had rested in an arm-chair for at least half an hour.

TABLE II.

Subject	Lactic acid in mg./100 c.c. blood			
M. M. M.	9·2	8·4	13·0	12·7
I. H.	16·6	12·4	14·0	13·5
E. I.	14·3	11·7	13·2	9·0
A. M. T.	10·4	9·6	10·7	10·7
F. J. S.	13·3	10·2	12·1	—
P. C.	14·5	9·0	—	—
J. C. B.	10·1	12·6	—	—
F. P.	10·3	14·6	—	—
K. C. H.	9·2	—	—	—

The values are lower than those of previous authors except Owles; a fact which suggests that they were obtained by an improved technique and afford a truer index of the lactic acid content of blood. Table II shows also that even with the same subject under apparently identical conditions the variations are considerable. It seems probable that these wide variations are due neither to experimental error nor to changes in the blood during or after shedding.

Possible factors influencing blood lactic acid production at rest.

(1) *Muscular contraction.*

It is known that lactic acid is constantly being produced in the muscles even during complete bodily repose. This was first shown in the case of frogs by Fletcher and Hopkins [1907] and amply confirmed by Meyerhof [1924] in the cases of amphibians and of mammals. It has usually been held that small amounts of the lactic acid so formed escape into the blood stream and that this supply constitutes the "resting" blood lactic acid. Against this hypothesis much evidence is accumulating. According to Hill, Long and Lupton [1924] the process of recovery after muscular exertion may be divided into two phases, the first being "nothing more than the oxidative removal of lactic acid in the muscles where it was formed before it has had time to escape into the blood and still further afield." This would appear to be the only phase necessary in very mild exercise, where lactic acid removal can keep pace with its formation. The second phase occurs when lactic acid formation is so rapid that some of it escapes into the blood stream, to be re-synthesized in the liver and in other muscles. Until recently the view that such moderate exercise as walking at rates of about 3½ m.p.h. produces a definite rise in blood lactic acid had not been questioned. This view depended mainly upon the work of Hill, Long and Lupton [1924], who found that a man walking at 3·5 m.p.h. for 28 min. raised his blood lactic acid from 20·9 mg. to 36·6 mg./100 c.c., whilst the same man walking at 4·1 m.p.h. for 33 min. raised it from 21·4 to 58·9 mg./100 c.c. Several other subjects were found by Long [1926] to give similar figures. Recent work by Owles [1930], however, has opened up the whole question as to the threshold at which lactic acid overflows into the blood after muscular exercise. Owles found in the case of two subjects in good training that walking for approximately 30 min. at speeds up to 4½ m.p.h. produced no rise in lactic acid in the blood taken from a superficial arm vein. At first sight these results suggested that, although no rise in lactic acid could be demonstrated in the blood from an antecubital vein, there might nevertheless be an escape of lactic acid from the leg muscles, but that the amount was insufficient to show a significant rise by the time it had been diluted in the general circulation and had passed through the inactive muscles of the arm.

In order to test the validity of Owles's results the following experiments were performed. A man of 48 (W. P. W.), who was accustomed to walking for about an hour every morning at a brisk pace (about 4½ m.p.h.), was made to walk at 4 m.p.h. for 30 min. Specimens of blood were taken from one of the antecubital veins at rest before exercise and from an antecubital vein and the femoral vein immediately after the exercise. The experiment was done on two separate occasions with identical results, which were corroborated in the case of another subject (B. T.), aged 55, who was accustomed to walking exercise and who walked for 30 min. at 4½ m.p.h. In neither case was there any rise in the lactic acid content of blood from the femoral vein, which demonstrates clearly that there was no escape of lactic acid into the veins immediately draining the muscles mainly involved in the exercise. Further experiments with

the subject B. T. showed that as soon as the rate of walking was accelerated to a speed incompatible with comfort, a rise in blood lactic acid occurred. Thus, after walking for 30 min. at 5¼ m.p.h., it rose from 10 mg. to 23·5 mg./100 c.c. In the case of men not used to strenuous walking, an increase of blood lactic acid was obtained after walking at lower speeds. The figures obtained in the above experiments are shown in Table III.

TABLE III.

Subject	Age	Blood lactic acid in mg./100 c.c. Arm vein, resting	Arm vein, after exercise	Femoral vein, after exercise	Rate and period of walking	Remarks
W.P.W.	48	16·9	17·8	16·9	30 min. at 4 m.p.h.	Good walker
		15·4	15·0	15·7	„ „	„ „
B.T.	55	11·3	11·8	10·8	30 min. at 4½ m.p.h.	„ „
		10·0	22·0	23·5	30 min. at 5¼ m.p.h.	Considerable effort needed to maintain this pace
M.S.	62	11·0	19·5	20·5	20 min. at 4½ m.p.h.	Fair walker for age
D.T.J.	24	10·5	20·5	21·0	24 min. at 4½ m.p.h.	Very poor training

It appears from these experiments that the threshold of exercise beyond which an increase of lactic acid appears in the blood depends on the "fitness" or "training" of the individual, a condition which is determined partly by the constitution of the individual, but mainly by accustoming the organism to exercise. Training, according to Briggs [1920], develops the efficiency of the mechanism which supplies oxygen to the tissues. As Owles suggests, the power of the muscles to eliminate *in situ* the lactic acid formed during exercise probably depends upon the adequacy of the blood supply to them.

In the light of the above results it is unlikely that the muscles are unable to eliminate *in situ* the relatively small amounts of lactic acid they produce during bodily rest. It is even more unlikely that the amounts produced in the same person under apparently identical conditions of rest should vary so much as to cause the large fluctuations actually found.

Further evidence against the probability of the lactic acid content of the blood at rest being due to muscular activity was supplied by taking specimens from the femoral artery and the femoral vein at the same time. In no case was there any appreciable difference between the venous and

arterial sample (Table IV). This indicates clearly that the passage of blood through a large group of muscles during rest causes no significant change in its lactic acid content.

TABLE IV.

Subject No.	Blood lactic acid in mg./100 c.c.	
	Femoral artery	Femoral vein
1	12·6	12·6
2	11·5	13·4
3	16·3	14·7
4	12·6	14·4
5	11·9	11·9
6	9·7	9·0
7	13·5	11·7

The value of these observations, however, would be considerably diminished unless a definite rise in the lactic acid content of the femoral vein over that of the femoral artery could be shown in conditions where the leg muscles were known to be producing large amounts of lactic acid, for example, after vigorous exercise. At first sight this might appear to be an easy matter, but when it is remembered how rapidly the blood comes into equilibrium and how great an increase of the circulatory rate occurs after severe exercise, it becomes clear that at best no more than a small increase can be expected.

Recovery curves after severe exercise of short duration, such as standing-running "all out" for 1 min., show that the blood lactic acid rises rapidly for a few minutes after the exercise and then falls very much more slowly, regaining approximately its resting level in about an hour. The more severe the exercise, the longer is the period of ascent of the curve, the higher the maximum point and the longer the recovery period. The maximum, however, is always reached within 5 or 6 min. At this point the arterial and venous blood are in approximate equilibrium, and in order to show any excess lactic acid in the blood of the femoral vein, simultaneous arterial and venous punctures must be made immediately after the exercise. In order to demonstrate this, standing-running "all out" for 1 min. was carried out in three cases. The subject was first stripped and the femoral area cleaned up in order to save time. As soon as the exercise was completed the subject lay upon a couch, and the left femoral artery and right femoral vein were punctured simultaneously by two operators. Withdrawal of the blood was delayed until both operators were ready, and was then accomplished at the same rate and to the same amount. In two cases the blood had been transferred from the syringe to the receiving vessel within 3 min. In the third experiment a delay occurred owing to the femoral artery being entered instead

of the vein. This necessitated a change of syringe and the blood was ultimately withdrawn about 5 min. after the exercise. The results of these experiments, although too few to be convincing, were satisfactory (Table V).

TABLE V. Simultaneous punctures of femoral artery and femoral vein after severe exercise.

Subject No.	Blood lactic acid in mg./100 c.c. Femoral artery	Femoral vein	Remarks
8	58·6	62·1	Within 3 min.*
9	71·8	74·8	Within 3 min.*
10	75·8	74·8	About 5 min.*

* After completion of exercise.

In Cases 8 and 9 the blood was taken within the period of lactic acid increase and a slight but definite excess in the venous specimen appeared. In Case 10 the blood was withdrawn approximately at the lactic acid maximum and showed no significant difference between the arterial and venous figures. The subjects of these experiments (Nos. 1–10) were chronic mental defectives or psychotics in good physical condition.

It has now been shown that no increase in blood lactic acid is caused by the passage of blood through a large group of resting muscles nor by the taking of a moderate amount of exercise, as long as it is not severe enough to produce dyspnœa or discomfort. This evidence clearly indicates the error of assuming that muscular activity is responsible for the blood lactic acid during bodily rest.

It has also been shown that the blood draining a group of muscles, which have been vigorously exercised, contains slightly more lactic acid than that of their arterial supply, provided that the lactic acid maximum has not been reached. This indicates the possibility of demonstrating changes of lactic acid in the blood occurring during its passage through muscle, but only when the formation of lactic acid is very rapid and before its removal has begun to keep pace with its formation.

(2) *Stimulation of the sympathetic nervous system.*

The effect of administration of adrenaline upon carbohydrate metabolism has been investigated in great detail, a review of the work being given by Cori [1931]. It has been demonstrated conclusively that injection of this substance leads to the breaking down of muscle glycogen without any associated muscular contraction, and that this results in the escape into the blood of large amounts of lactic acid.

The effects of subcutaneous injections on the blood lactic acid of man are shown in Table VI. It is known that excitement, fright or any emotion which powerfully stimulates the sympathetic nervous system

TABLE VI. Effect of 17 minims of 1/1000 solution of adrenaline hydrochloride given subcutaneously.

Subject	Blood lactic acid in mg./100 c.c.		*p*H		Pulse rate			
	Before adrenaline	20 min. after	Before	20 min. after	Before	10 min. after	20 min. after	30 min. after
F. J. S.	12·1	22·0	7·34	7·34	74	80	90	90
A. C.	13·5	24·8	—	—	No significant change			

increases the secretion of adrenaline with consequent rise of blood lactic acid. For this reason it might be suggested that the mood and emotional state of the individual, through their influence upon adrenaline secretion, are the regulators of the lactic acid content of the blood during rest. The present work, however, supplies little evidence in support of this view, since the blood lactic acid levels in the same subject appear to be entirely independent of mood. Furthermore, in experiments, not included in this paper, upon neurotic patients some of the highest levels were found in the most sluggish and phlegmatic of cases, whilst subjects showing sympatheticonia or suffering from anxiety states showed levels not appreciably above the average normal.

(3) *Lactic acid production in the brain.*

The production of lactic acid in relatively large amount by the brain tissue has been shown to occur both *in vitro* [Warburg, Posener and Negelein, 1924] and *in vivo* [Holmes and Sherif, 1932]. The possibility of some of this lactic acid overflowing into the blood and thus constituting a part of the resting blood lactic acid had therefore to be considered. Specimens of blood were accordingly collected from the jugular bulb and from the femoral artery. It is obvious that the blood in the main arteries does not vary significantly, and for this reason the femoral artery, being the most convenient to enter without delay, was chosen in preference to the carotid artery. As not more than 2 min. elapsed between the removal of the venous and arterial blood, the samples may be taken as simultaneous for comparative purposes. The subjects were in the resting state and were of the same type as those used for other arterial punctures.

Table VII shows that the lactic acid level of the blood directly issuing from the brain is no higher than that of the arterial blood, that is to say, that all the lactic acid formed in the brain is dealt with *in situ* and plays no part in forming the resting blood lactic acid. The free passage of CO_2 into the blood, on the other hand, is demonstrated by the differences between arterial and venous *p*H values.

TABLE VII.

Subject No.	Lactic acid in mg./100 c.c. blood		*p*H	
	Jugular bulb	Femoral artery	Jugular bulb	Femoral artery
11	12·3	12·6	7·34	7·37
12	18·0	16·2	7·33	7·36
13	12·2	14·0	7·35	7·38
14	17·2	15·4	7·31	7·35
15	16·4	14·5	—	—
16	10·2	11·7	—	—

(4) *Threshold for lactic acid resynthesis.*

The possibility of a threshold below which lactic acid is not resynthesized by the muscles would afford an attractive hypothesis, in that it would solve in an easy manner the problem of the source of blood lactic acid at rest. The existence of such a threshold value would also account for the fact that the resting levels of the blood supplying and leaving a group of muscles on the one hand and the liver on the other are all approximately the same [Himwich, Koskoff and Nahum, 1930]. It would not, however, account for the wide variations obtained and there is at present no practical evidence to justify the assumption of such a threshold.

(5) *Glycolysis.*

The conversion of blood sugar to lactic acid *in vitro* has long been an established fact. The disappearance of glucose in shed blood was noted by Claude Bernard [1877], and has since been studied by many workers. Lovatt Evans [1922] has shown that large amounts of lactic acid are produced by glycolysis in shed blood and that the reaction is accelerated by alkali. It appears that the phenomenon is due to some extent to leucocytic activity and that it cannot occur in the plasma or serum alone. It is known to be accelerated by moderate increases of temperature or by raising the blood *p*H, to be retarded by decrease in *p*H and to be stopped completely by the addition of sodium fluoride in suitable concentration.

The existence of glycolytic activity in the blood *in vivo* is more difficult to prove, but considerable evidence is accumulating in its support. Eggleton and Evans [1930] have demonstrated in a lung-limb preparation of a dog a fall of blood sugar coincident with a rise of lactic acid, caused by raising the *p*H of the blood by means of sodium bicarbonate. Still more convincing is the work of Anrep and Cannan [1923] who found that in a heart-lung preparation an increase in *p*H led to an increase in blood lactic acid. In this case there could be little production of blood lactic acid by muscular activity, since it has been shown by Himwich, Koskoff and Nahum [1928] that the heart is more concerned with removing than with forming lactic acid. Anrep and Cannan considered the increase in lactic acid to be due more to interference with its oxidative removal than to its formation from sugar. Against this, however, it has been shown by Hartree and Hill [1924] that in the frog a fall in *p*H tends to retard lactic acid removal and that a rise in *p*H has no such effect.

In the present investigation, the effect of alkali ingestion upon man was determined, 20 g. of sodium bicarbonate dissolved in water being given by the mouth. The results recorded in Table VIII show that 30 min. after ingestion a small but definite increase in both blood *p*H

TABLE VIII. Ingestion of 20 g. sodium bicarbonate.

Subject	Blood lactic acid in mg./100 c.c.		pH	
	Before	30 min. after	Before	30 min. after
J. C.	13·0	15·8	7·31	7·35
W. S.	11·7	14·4	7·30	7·35
A. M.	11·5	13·7	7·31	7·36
M. H.	16·8	20·1	7·32	7·37

and blood lactic acid could be demonstrated. On the hypothesis that glycolysis is the source of the "resting" blood lactic acid, these results supply one at least of the reasons for the variations of blood lactic acid found in the same resting individual at different times. It is known that slight changes in *p*H are constantly taking place in the circulating blood, and it is to be expected from the above evidence that these are accompanied by changes not only in CO_2 tension but also in lactic acid concentration. Thus glycolytic activity, stimulated by a tendency to increased blood *p*H and depressed by a tendency to decreased blood *p*H, offers a material basis for the suggestion of Anrep and Cannan [1923] that blood lactic acid changes serve as a third line of buffer resistance, and for the statement of Peters and van Slyke [1931] that (in states of alkalosis) the "lactic acid increase must be looked upon as an adaptive reaction, as it tends to mitigate the alkalosis to which it is a response."

SUMMARY.

1. The technique of lactic acid estimation by means of one of the more recent modifications of Clausen's method is described. It is shown that the removal of sugar is unnecessary as long as tungstate-sulphuric acid deproteinization is used.

2. An attempt is made to trace the source of blood lactic acid in man during bodily rest. Resting blood lactic acid values observed by previous authors are reviewed and are compared with the figures obtained in the present studies. The results show that any satisfactory explanation of the existence of this lactic acid must also account for the wide variations observed in the same individual at different times, but under apparently identical conditions of bodily rest.

3. Possible sources of blood lactic acid at rest are discussed and experimental evidence is presented in support of the following conclusions:

(*a*) During bodily rest the muscles supply no lactic acid to the blood. In the case of men in good training, walking at speeds up to 4½ m.p.h.

for 30 min. produces no increase in the lactic acid concentration of blood drawn from the femoral vein.

(*b*) Activity of the sympathetic nervous system with consequent secretion of adrenaline is an unlikely source.

(*c*) Comparison of blood samples taken in immediate succession from the jugular bulb and from an artery during rest shows that the blood receives no demonstrable amount of lactic acid from the brain.

(*d*) The existence of a threshold value below which lactic acid is not resynthesized by the muscles is an hypothesis which lacks supporting evidence of a practical nature and which would still leave unexplained the wide variations in lactic acid concentration.

(*e*) Conversion of blood sugar (glycolysis) is the most probable source of the blood lactic acid at rest. The evidence of glycolytic activity, shown by previous workers to take place *in vitro* and in heart-lung preparations, is supported by experiments *in vivo*, in which an increase in blood *p*H by means of alkali ingestion is shown to increase the blood lactic acid concentration. These experiments also suggest that the variations in lactic acid concentration during bodily rest are due to stimulation or depression of glycolytic activity and assist the organism in its endeavour to maintain a constant blood reaction.

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ADRENALINE IN THE SUPRARENAL MEDULLA.

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Of the many chemical methods which have been described for the estimation of adrenaline in extracts from the suprarenal glands, most depend on the use of oxidizing reagents to produce a red-coloured product of the partial oxidation of adrenaline itself. The method of Folin, Cannon and Denis [1912], on the other hand, depends on the blue colour produced in an alkaline phosphotungstate mixture by the reducing action of adrenaline. There is an obvious possibility that derivatives of adrenaline, not possessing its physiological activity, might, if present in an extract, contribute to the red colour produced by oxidizing reagents; while reducing substances other than adrenaline will produce a blue colour with Folin's reagent. It is not surprising, therefore, that several investigators have drawn attention to cases in which the colorimetric estimate of adrenaline in an extract differed significantly from that obtained by a physiological determination of activity; though, in general, the results obtained with extracts from fresh, normal glands have usually shown good correspondence, as in the original comparisons made by Folin, Cannon and Denis [1912]. Where discrepancies have occurred, these have usually been in the direction of over-estimates by the colorimetric test. Kojima, Nemoto, Sato, Saito and Suzuki [1932], on the other hand, recently drew attention to another kind of discrepancy, apparently due to the presence of other physiologically active substances in an extract, causing differences also between the physiological estimates obtained by different methods.

Such discrepancies as have hitherto been recorded are of different kinds, and of much smaller dimensions than that recently described by Annau, Huszak, Svirbely and Szent-Györgyi [1932]. These authors examined extracts from the separated medulla of ox suprarenal glands, rapidly frozen as soon as possible after slaughter, and kept in a frozen condition until the extract was prepared by a method designed to avoid decomposition of the natural hormone. In such extracts they observed a

physiological activity corresponding to that of about ten times the quantity of adrenaline estimated from the colour produced by the oxidizing action of iodine. The existence of the discrepancy was confirmed by other colorimetric tests. It could be interpreted as due either to the presence of some substance or condition hindering the oxidative production of the coloured derivative, or to the existence of adrenaline in the suprarenal medulla as an unstable derivative having many (at least 10) times the activity of the free substance, as isolated by chemical methods. Szent-Györgyi and his co-workers chose the latter interpretation, and provisionally gave the name "novadrenine" to this active derivative, in which they supposed adrenaline to occur naturally, as the true, unchanged suprarenal medullary hormone. Their choice appears to have been determined by two considerations. The physiological action of their medullary extracts, if due to free adrenaline, would have indicated the presence of as much as 15 to 30 mg. of adrenaline in 1 g. of fresh medullary substance. Apparently they regarded such a high content as unthinkable. Further, in attempting to isolate the supposed novadrenine, they found that the values determined by colour and physiological tests would, at some stage of purification, become newly concordant. They attributed this to liberation of adrenaline from the unstable novadrenine; but, in the absence of any quantitative data, to show that the new match was reached by loss of physiological action rather than by gain of colorimetric value, the fact recorded could as easily be explained by removal of hindrance to oxidative colour production.

The experiments here recorded were undertaken to obtain further evidence as to the meaning of the discrepancy described by Szent-Györgyi and his co-workers[1].

Methods.

Material and extraction. Szent-Györgyi and his co-workers laid great stress on the protection of the suprarenal glands from *post-mortem* change by cold, and on the use of what they regarded as a conservative method of extraction. Our precautions were probably at least as strict. They state that the glands (ox) were excised for them as soon as possible after death, and transported on ice to the laboratory, where they were frozen before dissection and mincing. We arranged to have ox glands from

[1] Since this paper was accepted U. S. v. Euler has published in this *Journal* (78, 462) an account of a shorter investigation, by which he also was led to conclude that the discrepancy in question had no true significance, being probably due to some defect in the colorimetric method used by Szent-Györgyi and his colleagues; and this conclusion has been accepted by Professor Szent-Györgyi, in a footnote to v. Euler's paper.

the abattoir removed as soon as possible, and frozen with solid CO_2 before transport to the laboratory. In other cases we were able to use glands from horses slaughtered at the Institute's farm; and these could be obtained within a few minutes of slaughter, and then similarly frozen for transport. We found no evidence, indeed, that this accelerated excision enabled us to obtain extracts of higher activity than those yielded by glands removed at the abattoir after a longer interval. In any case, however, our failure to confirm the suggested discrepancy between colorimetric and physiological values was not due to the neglect of precaution against *post-mortem* change. As a rule the hard frozen glands were dissected as soon as received in the laboratory; but in some cases they were kept for periods up to 2 weeks at about −4° C. before use. Glands so stored yielded as active extracts as those obtained from others of the same batch worked up immediately on delivery.

The frozen glands were cut open, and the medulla dissected as cleanly as possible from the cortex without allowing it to thaw. We did not use a mincer, but transferred the medullary substance to a mortar containing the cold extraction fluid and clean sand, with which the substance was quickly ground to a fine suspension. Three methods of extraction were used.

(i) Szent-Györgyi's method, of extracting with cold 0·5 p.c. trichloroacetic acid, raising the mixture briefly to 70° C. to complete the coagulation, and then cooling the filtered extract and preserving below 0°.

(ii) An alternative method, mentioned by Szent-Györgyi as equivalent, of extracting with 5 p.c. trichloroacetic acid in the cold. At this strength the cold acid itself is an efficient protein coagulant, and no heating is required. After filtration the excess of trichloroacetic acid can be removed, as suggested to us by Dr Dudley, by shaking with several successive quantities of ether, carefully purified from peroxides. The ether also removes traces of lipoid substances, which make the extract slightly opalescent and hinder colour comparisons. Shaking with ether is repeated until the extract is perfectly clear and colourless, and is only just acid to congo-red paper. The extract is then preserved below 0° C.

(iii) The familiar method of extracting with 0·5 p.c. acetic acid, boiling briefly to coagulate proteins, and filtering.

It will be seen later that the results obtained by the three methods of extraction are practically identical; so that our experience affords no ground for supposing that the method of extraction used by the Hungarian workers had any special effect in preserving a natural adrenaline complex.

Methods of colorimetric determination. With the exception of the Folin method, in which adrenaline acts as a reducing agent to produce a blue-coloured derivative of phosphotungstic acid, the different methods which have been described for the colorimetric determination of adrenaline depend on the formation of a red product of partial oxidation. Szent-Györgyi and his co-workers used a method of this kind, depending on the production of the red colour by oxidation with iodine, as originally described by Vulpian. They treated the extract to be tested and a control solution of pure adrenaline simultaneously with Lugol's solution, added in sufficient excess to colour the solution with free iodine; the free iodine was then removed by sodium thiosulphate and the colorimetric determination immediately made by direct comparison of the two solutions. The value obtained was controlled by other tests, including that based on oxidation by potassium persulphate, introduced by Ewins, and identical results are stated to have been obtained. In all cases the determination was based on direct comparison of the colour produced in the extract with that in a control solution of pure adrenaline.

In our own experiments the persulphate test was first tried, on account of the facility which it offered, by the use of a colourless reagent, for studying the course of the development of the colour in the two solutions. We were immediately struck by the extraordinary slowness with which the colour appeared in the gland extract. In the pure adrenaline solution the colour appeared rapidly, and the oxidation had passed far beyond the stage of maximum red colour at a time when the red colour was in the initial stage of its slow development in the similarly treated gland extract. No kind of estimate of the adrenaline in the extract could be obtained by direct comparison under such conditions. Means could be found, indeed, as described below, to reduce this disparity in time relations, but without any certainty of its elimination. There was a further hindrance to the accuracy of direct comparison, in the fact that the colour produced by partial oxidation of adrenaline, by any reagent, is a mixed colour, with a red, a yellow and a blue component, which may to some extent vary independently; so that, even if the process could be so modified as to produce certainly simultaneous colour maxima in the pure adrenaline solution and the gland extract, the direct comparison would still be liable to difficulty and inaccuracy on account of minor qualitative differences between the colours.

For these reasons we abandoned the attempt to make estimates by direct comparison in a colorimeter of the Dubosq type, with any of the colorimetric methods. Using the Lovibond-Rosenheim [1927]

tintometer, in which the colour of the solution is rapidly matched by an appropriate combination of standard red, yellow and blue glasses, we could determine, for adrenaline solution and for extract alike, the strength of the red component of the colour at its maximum. An accurate and reproducible comparison could thus be made, even when the maxima were not simultaneous. It was only necessary to choose conditions which would lead to the development of the colours in the two solutions at rates not too widely different, and of the different colour components in approximately the same ratios.

The three tests thus used by us were the persulphate test of Ewins, as modified by Barker, Eastland and Evers, the iodine test chiefly used by the Hungarian observers, and the Folin test.

The persulphate method. In a recent examination of the development of the colour in this test, Barker, Eastland and Evers [1932] showed that the rapidity of the development and the time of persistence of red colour were favoured by the presence of the chloride ion, by a *p*H between 5 and 6, and by the presence of a trace of a heavy metal, in particular of copper. Having confirmed these authors on these various points by our own experience, we have used, in all our own determinations by this method, a buffered persulphate reagent, made up in accordance with their indications, to the following formula, with a *p*H 5·5:

NaCl	1
$K_2S_2O_8$	0·2
$Na_2HPO_4, 12H_2O$	0·239
$NaH_2PO_4, 2H_2O$	0·937

Water to 100.

To the extract or control adrenaline solution the persulphate reagent was added in equal volume. The mixture, at the temperature of the laboratory (18°–20° C.), was placed in the tintometer and successive matches made with the coloured glasses until the maximum value for the red component was reached.

The sensitiveness of the reaction to traces of heavy metals makes it difficult to control the rate at which the colour develops. The use of a syringe with a steel needle, instead of a glass pipette, to measure a small volume of an extract, was found, on one occasion, to produce a great acceleration of the colour development. We tried the effect of deliberately accelerating the development by dipping a strip of clean copper into the mixture of extract and persulphate reagent, and found that the colour, as expected, reached its maximum much more rapidly, but that the

maximum so attained was somewhat lower than that ultimately reached in the same mixture without copper.

Apart from such known and deliberate changes of conditions, affecting the rate of colour development, we observed changes in the rate which appeared to be capricious, and were due to causes which we were unable to identify. We observed, on one occasion, that a freshly prepared medullary extract showed a very pronounced retardation of the development of maximum colour with the persulphate reagent, and that the inhibiting factor had, apparently, largely disappeared when the extract was tested again after being kept for a day or two, the same maximum being then attained, but with a rapidity comparable to that seen with pure solutions of adrenaline. Later, however, the same extract again showed a retarded colour development. In successive tests made on one extract, even at different times during the same day, widely varying periods were required for the attainment of the colour maximum. Attempts to associate these variations with changes in illumination or other conditions were unsuccessful. The reaction is extremely sensitive to traces of heavy metals, as already indicated; and this probably explains the fact, observed by us, that the colour development is greatly retarded by adding potassium cyanide to the persulphate reagent, in dilutions as low as one-fiftieth of the molar concentration of the persulphate. Even pure solutions of adrenaline are to some extent subject to apparently capricious variations in their rate of oxidation by this reagent; but these are much more prominent in dealing with gland extracts.

Whatever may be the sources of these irregularities, they appear to us to be of such a kind that there would be little hope of eliminating them, under the working conditions of an ordinary laboratory. When the time of maximum development of the colour in an extract may vary from 5 min. to an hour, and when the average time required by extracts to reach the maximum is about three times that required by a pure solution of adrenaline, it is clear that direct comparison of the colour developed in an extract with that of the control solution at its maximum can only give grossly misleading results. Under such conditions we should have found it easy to obtain measurements comparable to those of Szent-Györgyi and his colleagues, suggesting that an extract contains adrenaline in only about one-tenth of the quantity corresponding to its physiological action. On the other hand, although the proportion of yellow to red in the colour at its maximum tends to increase somewhat with the time of development, the readings of maximum value of the red component by the Lovibond-Rosenheim tintometer showed but slight variation

with the same extract, even over a fairly wide range of times taken in reaching the maximum. By comparison of these maximum red values with those of a pure adrenaline solution, satisfactorily consistent estimates of the adrenaline content of an extract could be obtained by the persulphate method. And these, again, agreed fairly well with the estimates obtained by the iodine method, when both were used for the same extract.

The iodine method. This was the method which gave the results on which the conclusions of Szent-Györgyi and his co-workers were mainly based. The course of the development of the colour in this reaction can be studied by adding iodine (Lugol's solution) in distinct excess to a series of samples of an adrenaline solution, and removing the excess of iodine with thiosulphate at different intervals after its addition. In the earliest stage the colour so revealed is a yellowish red, which, in samples from which the iodine is discharged later, becomes successively a pure red, and then a bluish red. The rate at which this sequence of changes occurs is conditioned by the hydrogen-ion concentration. At *p*H 6·2 the following sequence can be observed by the tintometer. If the excess of iodine is removed as soon as possible after addition, a colour is observed containing about 85 p.c. red to 15 p.c. yellow by the Lovibond glasses. If the excess of iodine is allowed to remain for 50–100 sec. before discharge, the colour obtained is a "pure" red—*i.e.* one requiring no admixture of yellow for matching in the tintometer—and the intensity of this is the same, within about 5 p.c., as that of the red component in the earlier mixture. If the iodine is allowed to act still longer, a blue component replaces the yellow in the resulting colour, while the red component still remains approximately constant. Finally, if the excess of iodine is allowed to act for 15 min. or more, both blue and red components diminish. In solutions more strongly acid than *p*H 6, the yellow component is stronger in the early state, and the development to the blue-red stage requires a longer action of the iodine. If the acidity is still greater (*p*H less than 4) the red colour develops very slowly. On the other hand, as the reaction is shifted to the alkaline side of *p*H 6, the sequence of changes is accelerated, becoming so rapid at a definitely alkaline reaction that it is difficult to be certain of stopping the reaction in the maximum range; and the colour, after discharge of excess of iodine, fades so rapidly in an alkaline mixture that it is not easy to obtain a stable and satisfactory reading[1].

A large excess of iodine in a strongly acid solution appears to destroy the red component faster than it is formed, so that a red solution is never

[1] Cf. v. Euler, *loc. cit.* p. 465.

obtained. At *p*H 6, on the other hand, a moderate excess of iodine makes no difference to the strength of the red colour obtained in the same time of action. At reactions more acid than about *p*H 6 the production of the colour maximum by iodine was always slower, though to varying degrees with different extracts, than with pure solutions of adrenaline. When, on the other hand, the reactions of both were in the neighbourhood of *p*H 6, there was no perceptible difference between the rates of colour production in a pure solution of adrenaline and in medullary extracts. In contrast with the persulphate test, and also with that of Folin, the rate of colour development in the iodine test appears to be unaffected by traces of heavy metals.

For these various reasons it was clear that a reaction in the neighbourhood of *p*H 6 provided the optimum conditions of colorimetric determinations by this method. Szent-Györgyi and his co-workers state that their extracts were "neutralized with a small excess of sodium bicarbonate." This would appear to indicate the choice of a reaction far too alkaline, in our experience, to enable stable and consistent readings to be obtained by the iodine method; but we are unable to judge to what extent this accounts for the difference between their results and our own. Our own procedure was as follows:

The control solution was made by dissolving an accurately weighed quantity of pure adrenaline base with the aid of a trace of dilute HCl, and diluting with distilled water. The reaction of the medullary extract to be tested was pre-adjusted, if necessary, to the neighbourhood of *p*H 6. A preliminary determination indicated the approximate strength of adrenaline to be determined. Appropriate dilutions of the control solution and of the extract were then made with a 1/15 molar phosphate buffer solution, made up according to Clark's directions, with a *p*H 6·2. Lugol's solution, diluted to a strength of *N*/100 iodine, was then added, in the proportion of 1 c.c. of the dilution to about 0·1 mg. of adrenaline. The mixtures were allowed to stand for equal times, in the range from 50 to 100 sec., experience having shown that within this range a stable colour was produced, which could be matched by the red glasses of the tintometer without introduction of a yellow or blue component. The determination can be made rapidly, and successive readings on the same solution or extract do not vary by more than $\pm$ 3 p.c. Since the colour develops at the same rate, under these conditions, in pure adrenaline solutions and gland extracts, and has the same pure quality in both cases, direct comparisons can also be made with a colorimeter of the Dubosq type.

Folin's test. In contrast to the other tests, in which adrenaline acts as the specific parent substance of the coloured product on which the measurement is based, Folin's test depends on the reducing action of adrenaline, in producing a coloured product of phosphotungstic acid. This reducing action is not specific. It is produced by physiologically inactive oxidation products of adrenaline, by uric acid, and, according

to Szent-Györgyi, by hexuronic (ascorbic) acid, which is a constituent of the suprarenal cortex.

There seem, therefore, to be many chances of the Folin test, when applied to suprarenal extracts, giving an estimate in excess of the true adrenaline content; and several investigators have stated, indeed, that extracts of the whole gland do give estimates higher than the true value [Lewis, 1916; Frowein, 1922; Maiweg, 1922; Watanabe and Sato, 1928]. On the other hand, a careful comparison made by Folin, Cannon and Denis [1912] at the time of the first description of the test, showed a very satisfactory correspondence between the adrenaline contents determined by it for a series of gland extracts, and the physiological estimates made on the same extracts.

Baker and Marrian [1927] have shown that the development of the colour in the Folin test takes a time which varies according to conditions, the presence of traces of heavy metals having, in particular, an accelerating action. In order to see the course of the development it is better to dilute the mixture of phosphotungstate and adrenaline-containing solution to near the final volume, before adding the sodium carbonate and thus starting the reducing action. It is then seen that the blue colour reaches about 85 p.c. of its maximum in about 3 min. The maximum is then more slowly approached, being reached only after about 10 min. Later, the colour slowly fades again. The colour is mainly blue, but the presence of a yellow component increases the difficulty of matching it in the Lovibond tintometer. On the other hand, the development of the colour is not delayed in the gland extract by the presence of non-specific constituents, so that direct comparison with the control solution, as in the ordinary performance of the test, gives good results.

Most of the criticisms of the test, as giving unduly high values through the presence of non-specific reducing agents, have been based on experiments with extracts from the whole suprarenal gland, including the cortex. We were dealing with extracts from the separated medulla, and these were so prepared as to be free from products of the partial oxidation of adrenaline. On the other hand, it is impossible to be certain of removing every trace of cortical material in dissecting out the medulla from the frozen glands. It might, accordingly, have been expected that our estimates on these extracts by the Folin test would give rather high values. As a matter of fact, it will be seen that, when the Folin test was applied, the estimate of adrenaline which it gave was consistently lower than that obtained on the same extract by the iodine method, and

usually lower than that given by the persulphate method. We are unable to explain the discrepancy, and can only record it.

Physiological determinations. For these we used only the test on the arterial blood-pressure reaction of the spinal cat. The cord was cut under ether at the level of the second cervical vertebra, and the cord anterior to this section, the bulb, and the whole of the brain completely destroyed. The determinations of the adrenaline content of an extract were made in the usual way by finding a dose producing a rise of arterial pressure matching, as exactly as possible, that produced by a submaximal dose of a control solution of pure adrenaline. The most favourable conditions for accuracy were presented by a preparation in which, some hours after cord section, the "resting" arterial pressure had attained a low, steady level. By bracketing doses the adrenaline activity of an extract could usually then be estimated to ± 5 p.c.

The general nature of the results soon became clear. When injections were, in the ordinary way, made into a peripheral vein, the adrenaline determined in an extract, in comparison with a pure adrenaline solution, was definitely in excess of that determined by any of the colorimetric methods used by us. The difference, indeed, was not of the order suggested by Szent-Györgyi and his co-workers. They found an activity at least 10 times as great as that indicated by the colour test. We found an excess activity corresponding to only 10–40 p.c. of the adrenaline determined by colorimetry; but it was generally present. An inspection of the arterial pressure records showed, however, a qualitative as well as a quantitative difference between the changes produced by equicolorimetric doses of pure adrenaline and of the gland extract respectively. The curve shows, in either case, a very rapid rise to an obvious "shoulder," from which the pressure climbs more slowly to the brief maximal "pinnacle." It could frequently be seen that the excess of the maximum rise produced by the dose of extract, over that produced by the colorimetrically equivalent dose of pure adrenaline, was entirely due to the second phase of the rise—a higher "pinnacle" being reached from an equally high, or even a lower "shoulder." Since this second phase involves, and is partly due to acceleration and augmentation of the heart beat, it seemed possible that some other constituent of the complex extract, by a reinforcing or steadying action on the heart beat, might assist in raising the blood-pressure to a level above that to which it would be driven by the unmixed action of the adrenaline present in the dose. This possibility could be tested, by injecting the doses of adrenaline and of gland extract into the thoracic aorta, so that they would pass

directly to the peripheral arterioles and produce their main pressor effects by vaso-constriction, effects on the heart being reduced to those of such traces of adrenaline or other substance as might pass through the peripheral vessels, and delayed by a whole circulation time. For this purpose it was necessary to use a long capillary glass cannula, introduced through the cut central end of the right carotid artery, and pushed down so that its tip rested against the inside of the lesser curvature of the aortic arch, as described by Dale and Richards [1927]. The blood had to be made incoagulable by heparine, and this involved a modification of the method of destroying the brain. By a preliminary dissection under ether both carotid arteries were tied, and then, the upper part of the sternum being split without opening the pleuræ, the vertebral arteries were tied in succession, and the subclavian arteries central to the origin of the vertebrals. The tying of the second vertebral artery was followed by a few deep gasps preluding the complete cessation of breathing. Artificial respiration being applied, the cord could now be cut, and the brain destroyed, as a completely bloodless operation. After all further dissection had been completed, and arrangements made for inserting the capillary aortic cannula, heparine could then be injected without any fear of hæmorrhage from the intracranial vessels.

Results.

The results are assembled in Table I, which enables the estimates obtained by the different colorimetric and physiological methods to be compared.

Table I. Mg. of adrenaline per g. of fresh, moist medulla.

		Colorimetric			Physiological	
Date	Animal	Iodine	Per-sulphate	Folin	Intra-venous	Arterial
Dec. 23rd, 1932	Horse	(*a*) 8·8	—	—	9·87 9·49	—
Jan. 2nd, 1933	Ox	(*a*) 5·68	5·4	5·12	7·33 7·74 7·33 7·94	5·12
Jan. 16th, 1933	Ox	(*a*) 8·43 (*b*) 8·18 (*c*) 7·33	—	—	12·0	6·74
Jan. 27th, 1933	Ox	(*a*) 9·86	—	8·88	12·28	7·9
Feb. 2nd, 1933	Ox	(*a*) 6·85 (*b*) 8·09				
Feb. 15th, 1933	Horse	(*a*) 10·74 (*b*) 11·47	10·74 10·50	9·81 9·93	10·74 12·62	

The following points may be noted:

(1) When colorimetric determinations are made by more than one method on the same extract, the results are in fairly good agreement, but the estimates by the Folin method are, on the whole, lower than those by the iodine method.

(2) The samples used on January 2nd and 16th were prepared from a batch of material collected on the former date. It will be seen that the extract made on the day of collection was much weaker, by all tests, than that made from other glands of the same batch, kept for a fortnight before extraction. Probably the glands chosen for immediate extraction happened to have smaller stores of adrenaline; in any case there seems no reason to doubt the stability of glands kept below 0° C.

(3) The three different extraction methods above described were used in certain cases. In Table I (*a*) indicates an extract made with cold 5 p.c. trichloroacetic acid, the excess being removed from the filtrate by ether; (*b*) an extract made, by Szent-Györgyi's usual method, with 0·5 p.c. trichloroacetic acid and brief heating to 70°; (*c*) an extract made by brief boiling with 0·5 p.c. acetic acid. It will be seen that there is no regular excess of adrenaline shown by any one process over the other when used on samples from the same batch of glands. Whenever the comparison was made, the extract by simple boiling with dilute acetic acid showed, further, a similar excess of activity by intravenous injection over the colorimetric determination to that shown by the extracts made from the same batch by either of the trichloroacetic acid methods recommended by the Hungarian workers. There seems no ground, in any case, for the suggestion that activity, as distinguished from adrenaline content, is lost by the simple, ordinary method of extraction.

(4) The adrenaline contents determined by the intravenous physiological test range from 7·33 to 12·62 mg. per g. of fresh medulla. None of these values reaches the range of 15–30 mg. per g. mentioned by Szent-Györgyi and his co-workers. On the other hand, the values are not of a lower order. According to their statement, their colorimetric values were not more than one-tenth of the physiological. Their colorimetric estimates, accordingly, showed not more than 1·5–3 mg. per g.; and these values are more definitely inferior to those obtained in our colorimetric determinations (5·7–11·5) than are our physiological determinations to theirs.

(5) Our physiological estimates, obtained by matching the heights of blood-pressure rises with intravenous injections, almost always showed a definite excess over the colorimetric estimates on the same extracts, as

Fig. 1a. Experiment of January 11th. Comparison of diluted extract (*E*) with equicolorimetric solution of adrenaline 1 : 50,000 (*A*), on spinal cat with venous injections. 0·35 *E* > 0·5 *A*. 0·3 *E* = (approx.) 0·5 *A*.

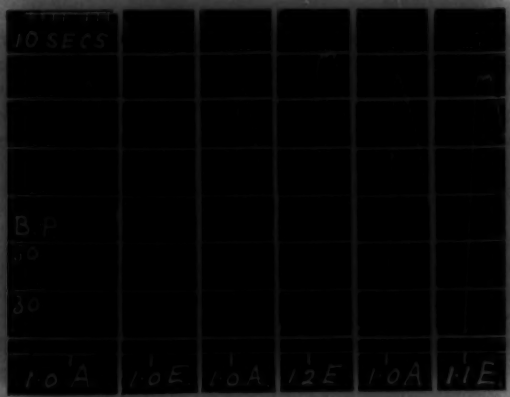

Fig. 1b. Continuation of experiment, with arterial injections into the same cat.
1·0 *A* > 1·0 *E*
< 1·2 *E*
= 1·1 *E*. ∴ 0·55 *E* = (approx.) 0·5 *A*.

has been mentioned already. The experiment of February 15th shows that the discrepancy is not always present. The discrepancy is, in any case, of a relatively small order, and has no relation to a difference of the kind described and illustrated by Szent-Györgyi and his co-workers. The estimates made by us on January 2nd, 16th and 27th with intra-arterial injections show a reversal of the discrepancy, the physiological estimates by this method being, in all cases but one, as definitely inferior to those made by colorimetry, as are the latter to the physiological estimates with ordinary intravenous injection. Fig. 1 is taken from part of the record representing the comparisons made on January 2nd.

Discussion.

The discrepancy which we have observed has no connection with that which we set out to investigate. The fact that it appears in opposite directions, according as the physiological tests are made with one or another form of injection, may be taken to indicate a limit to the accuracy with which the actions of a crude extract and of pure adrenaline can be compared. The comparison is clearly subject to interference by some substance which potentiates the pressor effect of adrenaline with intravenous, and antagonizes it with intra-arterial injections. The simplest conception is of a substance, or mixture of substances, which—at least when adrenaline is also present—improves the output of the heart by a direct action on that organ, and has also a peripheral vaso-dilator action, antagonistic to the vaso-constrictor effect of adrenaline. A comparatively very weak activity of this kind would suffice to modify the adrenaline effect in opposite directions with the two methods of injection, so as to produce the disparity between the two estimates, and between either and the colorimetric estimate, which presumably gives the nearest approximation to the true adrenaline content, at a value somewhere between the two physiological estimates.

We find no evidence at all for the existence of a real, major discrepancy of the kind described by Szent-Györgyi and his colleagues. It seems probable that the low colorimetric values which they obtained were due to various conditions which cause the coloured product of partial oxidation to appear more slowly in the impure extract than in the pure adrenaline solution, or which otherwise produce a false reading of the adrenaline content when direct comparison is made between the simultaneous colorations of the two solutions. Our results, it will be seen, eliminate the large discrepancy, not by depressing the physiological estimates to meet colorimetric values as low as theirs, but by raising the

colorimetric values, determined under fairer conditions by reading each at the maximum, to meet physiological estimates of the same order as theirs. The highest value we obtained, by physiological assay, was, indeed, not much above 12 mg. of adrenaline per g. of fresh medulla. We find no reason to doubt, however, that, with certain samples of glands, we should obtain extracts of a physiological activity corresponding to 15 mg. or more of adrenaline per g., or that colorimetric tests, properly applied, would show that activity to be due to adrenaline itself. Adrenaline contents of this order in the separated medullary substance are not, as Szent-Györgyi and his co-workers have assumed, "impossible." The tenfold discrepancy observed by them was clearly due, in our opinion, to their underestimating, to that extent, the amount of adrenaline actually present, and not, as they supposed, to its presence as novadrenine, with an activity at least ten times as great.

SUMMARY.

The existence of adrenaline in the suprarenal gland as a complex, novadrenine, of enhanced activity, is not confirmed. Adrenaline exists in the gland in a quantity corresponding to the physiological estimate.

I wish to express my sincere gratitude to Sir Henry Dale for his continual help and advice during this research.

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THE INFLUENCE OF CERTAIN FACTORS UPON THE VOLUME OF THE INTRATHORACIC VENÆ CAVÆ.

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It is probable that many factors affect the volume of the intrathoracic venæ cavæ of mammals during respiration; in this paper only two will be considered in detail, namely, structure and intramediastinal pressure. The thoracic inferior vena cava of the dog, cat, and rabbit [Franklin, 1931, 1932, 1933] lengthens during inspiration, but the superior vena cava does not generally do so in quiet breathing, although its structure in the cat and in the rabbit would permit such lengthening if it became necessary. If the thoracic inferior vena cava reaches its limit of lengthening, the heart is pulled caudally and the trachea and superior vena cava lengthen a little; inspiration, however, is becoming rather forcible when this stage is reached. The structure of the thoracic inferior vena cava in the dog, cat, and rabbit imposes variations in volume according to its length, and when other factors, such as respiratory variations in intramediastinal pressure, are excluded, the volume is found to be greatest at the expiratory length of the vessel. If, however, as is generally accepted, the intramediastinal pressure decreases during inspiration, this will tend to increase the volume of the vein and so will oppose the effect produced by its structural peculiarities. The primary object of the present research has been to investigate the combined effect of these two opposite influences upon the vein volume. At the same time, the volume changes of the dog's superior vena cava, when exposed to varying pressures, have been recorded. The structure of the latter vein in this species gives no anatomical justification for supposing that it undergoes functional changes in length; it differs, therefore, in this respect from the corresponding vessel in the cat and in the rabbit.

Many difficulties have arisen during the work, and of these two in particular must be mentioned here. The first is that it is not easy to obtain from the literature any satisfactory quantitative conception of the intramediastinal pressure changes in different species of mammals. It is very

doubtful if "intrapleural" pressure records can give any indication of intramediastinal values [Rehfisch, 1927], and even van der Brugh's technique [1900] leaves one somewhat sceptical. Meltzer [1892] essayed the measurement of intramediastinal pressures in large rabbits, and he found that the pressure changes were extremely small except at the caudal end of the mediastinum, so that only the diaphragmatic end of the thoracic inferior vena cava was exposed to pressure variations of any magnitude. From a study of all the available data, one gathers the impression that the variations in pressure within the dog's mediastinum during ordinary breathing are very unlikely to be as great as 20 cm. H_2O, and are probably very much less, especially at the cranial end of the thorax. The second difficulty of importance has been the peculiar nature of the thoracic inferior vena cava itself. Structurally, it is very largely non-cellular, as it consists for the most part of collagen and elastic fibres. It changes in length, as the diaphragm contracts, many thousands of times during the day. It has a scanty endowment of *vasa vasorum*. It is, therefore, a vessel *sui generis*, and in consequence it is not so surprising that the isolated vein deteriorates rapidly, and that diffusion often takes place through its walls, though the superior vena cava does not show corresponding permeability. The experiments on this point are being continued, and are only referred to here in order to explain why recording has had to be extremely rapid, once the vein has been removed from the body.

Method.

The volume variations of the isolated superior venæ cavæ and thoracic inferior venæ cavæ of six dogs were recorded by means of a special apparatus. The superior venæ cavæ were kept at their natural length, and were exposed rapidly to internal pressures of Ringer's solution varying from 0, 2, or 4 cm. to 30 cm. and back again. The inferior venæ cavæ were exposed to similar pressure changes from 4 or 6 cm. Ringer's solution to 30 cm. and back again, but this was done at both the expiratory and inspiratory lengths of the vessels, and usually also at intermediate lengths. The volume at each 2 cm. difference of pressure was automatically recorded by an optical device, and a complete cycle from low to high pressure and back took 2–4 seconds to record, so that the vein was exposed to the pressures for about the length of a normal respiratory cycle. Increase in internal pressure within the vein was assumed to be equal in its effect on the vein volume to a decrease in external pressure, such as would occur in the mediastinum of the intact animal.

The front and back views of the apparatus used are pictured in Figs. 1 and 2 respectively. A strip of teak *ab*, with another piece of teak *c* fastened on to it at right angles, is pivoted at *a* in a five-ply board *d*. The pivot is formed by one horizontal arm of a four-way metal tube *e*, which is firmly attached to the teak strip *ab*. Into the lower end of this metal tube is sealed a glass tube *f* of suitable calibre, which is held further down by the screw collar *g*. The lower part of the glass tube is bent round parallel to the teak strip *c* and has a flanged end. A second glass tube of

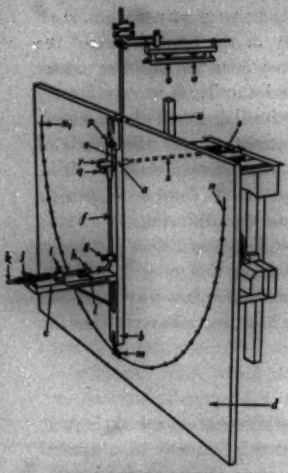

Fig. 1. Front view of apparatus described in text.

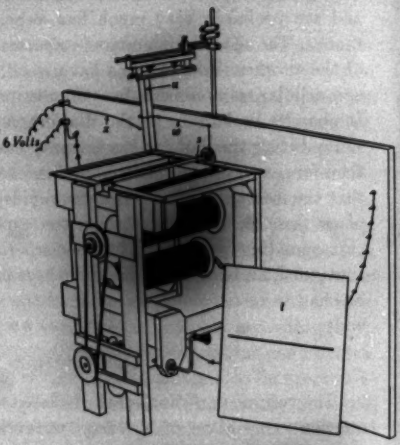

Fig. 2. Back view of apparatus described in text.

the same calibre, with one end *h* flanged and the other end *k* closed, is held in position by a screw collar *i* and can be moved backwards and forwards without rotation by a special device at *j*. An adjustable pointer on the glass tube *hk* indicates on a millimetre scale *l* the distance between the flanged ends of the glass tubes. A copper shoe *m* on the end of the teak strip *ab* is connected by a wire to the metal pivot tube, and makes contact with a series of thirty-one metal studs between *n* and n_1. These studs are all wired together at the back of the board, and are so arranged that they flash on the 4 volt bulbs *oo* at 2 cm. pressure intervals as the shoe passes round the semicircle from *n* to n_1. There are two taps on the four way

metal tube *e*; *p* is opened to let out air or fluid, *q* controls the socket *r*. The back view of the apparatus (Fig. 2) shows a detachable pipette *s*, which fits tightly, by means of an india rubber collar, into the pivot arm of the four way metal tube. The left-hand end of the pipette in the figure is open to the air. There are four rollers, shown in continuous black; the two lower ones are of wood with metal flanges, the two upper ones of brass. The gramophone motor indicated in the figure drives the upper wooden roller, and photographic paper (12 in. Kodak "Nikko" bromide soft) is thereby wound off the lower motor roller, and in its course passes over the left-hand brass roller, under the pipette, and over the right-hand brass roller onto the upper wooden roller. When records are being taken, the metal plate *t* is placed in position above the brass rollers and below the pipette, and the central slit in it lies immediately under the pipette. The lever *u*, when pushed to the left in Fig. 2, starts the motor and switches six volts into the lighting circuit, so that the lights flash on whenever the shoe *m* touches one of the metal studs in the semicircle nn_1.

The isolated vein is tied over the flanged ends of the glass tubes and the tube-vein-tube-pipette system is filled through the free end of the pipette with Ringer's solution at appropriate temperature. While this is being done, tap *q* is kept shut and tap *p* is left open until all air has been expelled. Then tap *p* is shut, a syringe inserted into socket *r*, tap *q* opened, and the meniscus of fluid in the pipette adjusted to a suitable point by withdrawal of fluid into the syringe. Tap *q* is then shut and the syringe removed. Rotation of the teak strip *ab* round the semicircle from *n* to n_1 causes the vein to be exposed to internal pressures of Ringer's solution from 0 to 30 cm. and back again, and a photograph of the meniscus in the pipette is taken, at every 2 cm. pressure difference, upon the paper moving underneath the slit. The rotation of the teak strip is done by hand in red light in a thermostat room at 37° C.

Results.

Dogs. As the technique varied very little, one experiment only need be cited at length. First, the apparatus was put into the thermostat room. Then a dog weighing 14 kg. was tied down on its left side, and the normal extent of its respiratory movements noted. A subcutaneous injection of atropine sulphate was made, and thereafter anæsthesia was induced with ether and chloroform. The skin over a portion of the left hind limb was incised and a venous cannula inserted into a vein thus exposed. Nembutal "844" sterile solution, in the dosage of 1 c.c. per 5 lb. body weight, was

injected through the cannula over a period of eight minutes, the general anæsthetic being discontinued after the first two or three minutes of the injection. A tracheal cannula was next inserted, and artificial respiration was begun. The skin over the right thoracic wall was reflected and, during a temporary cessation of artificial respiration, a large portion of the wall was removed. Then artificial respiration was resumed for some minutes. In the meantime a clip was attached to the diaphragm by the side of the caval foramen; to this clip was fastened a string and to the string a rubber band, looped over a nail on a centimetre scale. When artificial respiration was again discontinued, the diaphragm resumed its contractions, and the excursion of the string-rubber junction was noted on the centimetre scale at a time when the respiratory movements were approximately as great as those previously observed in the intact animal. The excursion of the whole thoracic inferior vena cava, so determined, was about 15 mm. Two points on the surface of the vein were then marked lightly with a cautery, and the distance between them measured at the expiratory length of the vein; this was 44 mm. The length of the superior vena cava was also measured between two fixed points. The thoracic inferior vena cava was cut out of the body, and tied on between the glass tubes of the apparatus at less than its expiratory length. Ringer's solution was run in, the vein adjusted to its expiratory length, and the meniscus in the pipette brought well away from the free end with 30 cm. solution pressure inside the vein. A quick test was made without a photographic record being taken, and it was found that the volume of the vein at 44 mm. length and 4 cm. Ringer's solution pressure equalled the volume at 54 mm. length (moderate inspiration) and 20 cm. pressure. The length of the vein was again adjusted to 44 mm. and three photographic records were taken at pressures from 4 to 30 to 4 cm. Ringer's solution, and at lengths of 44 mm., 54 mm., and 57 mm., the last being the observed inspiratory length of this piece of the total vein. When the records had been taken, the volume of the vein at 57 mm. length and 30 cm. pressure was measured by filling the whole system, sealing the free end of the pipette, and withdrawing the fluid in the vein through the tap *q* (Fig. 1) by means of a syringe inserted into the socket *r*. This volume was 5·0 c.c. Then the superior vena cava was tied on at its normal length, and a record taken of its volume changes with pressures of Ringer's solution from 2 to 30 to 2 cm. Its volume, when measured at 30 cm. pressure, was 6·5 c.c., but the whole length of the vein was not used.

The results are shown graphically in Fig. 3. (The kink in curve I was due to the screw-collar at *s* being a little too slack, and was not due to the

vein in any way. Recording is best done rapidly, and it is not easy to alter the length quickly if this screw collar is too tight.) It seems probable, from the graph, that the volume of the thoracic inferior vena cava diminishes a little in inspiration. The curve for the superior vena cava was plotted from the averages of the double readings.

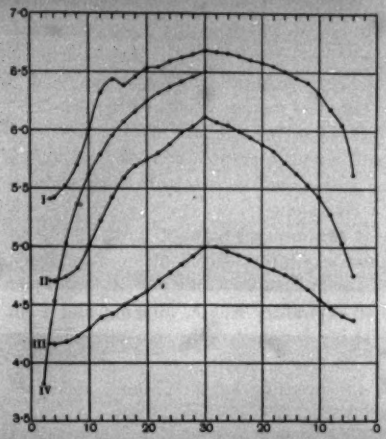

Fig. 3. Graph showing the volume of the isolated thoracic inferior vena cava of a dog at different lengths and pressures, and of the isolated superior vena cava of the same dog at different pressures. Curve I, inferior vena cava at expiratory length, 44 mm.; Curve II, at 54 mm.; Curve III, at 57 mm. (observed inspiratory length). Curve IV, superior vena cava. Ordinates: volumes in c.c. Abscissæ: pressures in cm. Ringer's solution.

The results with the veins from the other five dogs were similar in character to those already described. In the case of the thoracic inferior venæ cavæ, they were in brief:

(1) Volume at expiratory length, 42 mm., and 6 cm. pressure, was 5·7 c.c. Volume at inspiratory length, 52 mm., and 30 cm. pressure, was 5·3 c.c.

(2) Volume at expiratory length, 36 mm., and 6 cm. pressure, was equal to the volume at inspiratory length, 44 mm., and 30 cm. pressure.

(3) Diffusion was seen to be taking place through the wall of this vein during the experiment, but even so the recorded volume at expiratory length and 20 cm. pressure was equal to that at inspiratory length and 30 cm. pressure.

(4) Volume at expiratory length, 45 mm., and 4 cm. pressure, was greater by 14 p.c. than the volume at 53 mm. length and 30 cm. pressure.

(5) Volume at expiratory length, 43 mm., and 9·5 cm. pressure, equalled the volume at 53 mm. length and 30 cm. pressure.

From the intrapleural pressure records in the literature, one would gather that the changes in the intramediastinal pressure are likely to be much less than those necessary in the above experiments to equalize inspiratory and expiratory volumes, i.e. this vein in the dog probably decreases in volume in inspiration, if structure and intramediastinal pressure are the only two factors which are concerned.

Cats. The technique was similar, except that tubes with smaller flanges were used, no photographic records were taken, and only the thoracic inferior venæ cavæ were tested. The results were even more marked than the canine ones:

(1) Volume at expiratory length, 30 mm., and 4 cm. pressure, was 0·84 c.c. Volume at inspiratory length, 37 mm., and 30 cm. pressure, was 0·68 c.c.

(2) Volume at expiratory length, 30 mm., and 4 cm. pressure, was 0·8 c.c. Volume at inspiratory length, 40 mm., and 30 cm. pressure, was 0·72 c.c.

(3) Volume at expiratory length, 27 mm., and 4 cm. pressure, was 0·75 c.c. Volume at inspiratory length, 35 mm., and 30 cm. pressure, was 0·68 c.c.

Rabbit. Only one rabbit was used. The volume of its isolated thoracic inferior vena cava at expiratory length, 28 mm., and 4 cm. pressure, was 0·91 c.c., at inspiratory length, 33 mm., and 30 cm. pressure, was 0·87 c.c.

In all the dogs, and in at least one of the cats, there was a band of diaphragmatic muscle passing over the inferior vena cava at the caval foramen. This has been noted before [Franklin, 1933] as worthy of more careful investigation, for it might constrict the vein at this point in inspiration. In one of the dogs it certainly did so, but further work is necessary before any general statement can be made, and only this passing reference will be inserted here.

As a control to the experiments recorded above with the isolated vein, Dr Robert Janker (of the Röntgeninstitut der chirurgischen Universitäts—Klinik und Poliklinik, Bonn) kindly undertook to make for the writer an X-ray cinematograph film. X-ray opaque substance (Perabrodil or Thorotrast) was injected into the femoral veins of four cats and a record taken in each case of its progress along the inferior vena cava to the heart. In Figs. 4 and 5 a thoracic inferior vena cava is shown in expiration and inspiration respectively, and it is quite clear that the

Figs. 4, 5. X-ray cinematograph pictures showing the thorax of a cat in the expiratory (Fig. 4) and inspiratory (Fig. 5) phases of a respiratory cycle. X-ray opaque substance in the inferior vena cava, showing black in the photographs. *I.V.C.* Inferior vena cava. *V.C.* Vertebral column. *D.* Anterior edge of diaphragm shadow. *H.* Heart.

volume of the vein is less in the latter phase. From the results of the writer's work, it is certain that one of the factors causing this diminution in volume is the structural peculiarity of the vein and its lengthening in inspiration. When the film is projected, it leaves no doubt in the mind of a spectator about the correctness of the views stated above, and thereby tends also to justify the suggestions, made elsewhere in this paper, about the intramediastinal pressure variations.

Discussion.

Haller [1756, 1760, 1762] was the first to investigate in detail the effects of respiration upon the veins and upon the movement of the blood within them. His general conclusion [1762, p. 300] was that in inspiration the blood was dammed back in the abdominal inferior vena cava and flowed in more freely from the superior vena cava, in expiration the opposite. In inspiration, also, owing to the increased volume of the vascular bed in the lungs, blood flowed more freely into these organs from the heart, and into the heart from the vena cava. It is to be remembered that in most of Haller's experiments no narcotic was used, and doubtless in many cases the respiration was excessive and dyspnœic. Under such circumstances the thoracic inferior vena cava would lengthen to its maximum in inspiration, and it is highly improbable that the great diminution in volume of the vessel, which its structure would tend to impose with this degree of lengthening [Franklin, 1931, 1932, 1933], would be counterbalanced by the effect of the decrease in intramediastinal pressure (Fig. 3). Haller found that the inferior vena cava between the kidneys and diaphragm emptied itself and collapsed in inspiration, while in expiration it filled up and became round. He also noted a back-wave of blood or of injected air in inspiration in this portion of the vein. Haller's results can be understood if the volume of the thoracic portion of the vein and its lumen become smaller in inspiration, for at the onset of this phase some of the blood in the vein would be pushed backwards towards the abdomen, some towards the heart, and thereafter the inflow would tend to be less through the narrowed tube. Hence, after an initial extra inflow of blood from this vein into the heart, the inflow from the superior vena cava would be easier and this vein might itself be increasing slightly in volume. In this connection Haller's experiment on a suckling pig [1756, p. 184] is of very great interest, because the respiratory oscillations in the tributaries of the superior vena cava continued in their entirety even after the chest had been opened on one side. This would rule out intramediastinal pressure changes.

Since the beginning of the nineteenth century [Barry, 1826] the conception of these pressure changes has gradually come into physiology, and theoretical considerations of their effects upon the intrathoracic veins have replaced anatomical and experimental investigation.

It is now suggested that the volume of the thoracic inferior vena cava decreases in inspiration in those mammals, in which this vein lengthens in inspiration, and has a particular structure [Franklin, 1931, 1932]. The dog, the cat, and the rabbit are among such mammals, the guinea-pig is not. But the guinea-pig breathes so rapidly that its fluctuations of intramediastinal pressure are probably very small, and in any case they can only act for fractions of a second, so the volume changes are probably insignificant.

The superior vena cava in most mammals will tend to increase slightly in volume and perhaps also in calibre in inspiration, but from all the evidence one would imagine that such increase is small.

In man the thoracic inferior vena cava is of small length and volume, and probably undergoes little variation in volume. Keith [1902], however, has suggested that the hepatic portion of this vein may be compressed during inspiration between the right crus of the diaphragm and the liver. If Keith's idea is correct, and one cannot accept in their entirety anatomical findings unchecked by physiological experiment, then diaphragmatic contraction would produce in man, by slightly different means, an effect similar to that which it produces in the dog, cat and rabbit. Keith's view would also explain Mosso's findings [1884].

On the whole, then, it would seem that respiration has little effect upon the total volume of the two intrathoracic venæ cavæ taken together. The great increase in blood volume within the thorax, which occurs in inspiration, must be due to increase in volume of the heart (Janker's films have shown that this dilates) and of the lungs.

Summary.

Experiments on the isolated veins of the dog, cat, and rabbit, lead one to believe that the thoracic inferior vena cava decreases somewhat in volume in inspiration, and that the superior vena cava increases somewhat in volume in the same phase of respiration. That the volume of the cat's thoracic inferior vena cava does decrease in inspiration is shown by X-ray photographs taken by Dr Janker.

I wish to thank Prof. J. A. Gunn for the interest he has shown in this work; to our laboratory staff, especially to Mr Tuckey, I am indebted

for ready co-operation at all times. I also wish to acknowledge help given to me in the conduct of experiments by Mr B. B. Hickey, of University College, Oxford. The work has been made possible by the generosity of the Rockefeller Foundation, and forms part of a study of the venous return in mammals; I wish here to record my great gratitude to the Foundation for its support. Finally, I should like to thank Dr Janker for making the X-ray film of the cats, and Prof. I. de Burgh Daly for many references to work on intrathoracic pressures.

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612.217:612.339

EFFECT OF BREATHING ON THE INTRA-ABDOMINAL PRESSURE[1].

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The greater part of the work on this subject has been done in regard to the pathology of enteroptosis. Keith [1907], in an article on this condition, gives figures for the pelvic and intra-gastric pressures found by himself and other observers. In observations made in 1895[2] he found that in the erect posture the pressure just below the diaphragm varied in an adult man from +4 to +8 mm. Hg, the rectal pressure from +20 to +28. In the recumbent posture the intra-gastric pressure rose to 16 mm. Moritz [1895] found a rectal pressure of 28 mm., the part of this due to the superincumbent weight of the viscera being 20 mm., the part due to the tonus of the abdominal muscles (gastric P) 8 mm. Schwerdt [1896] observed a rectal pressure of 35 mm. of which 27 were due to gravity. These observers agree in finding a rise averaging 4 mm. during normal inspiration with a fall to the original pressure in expiration.

Some observers have found a negative intra-gastric pressure during deep inspiration. Keith ascribes this to the fact that their subjects suffered from the loss of abdominal muscular tone seen in enteroptosis in which condition the sub-diaphragmatic pressure may be constantly negative, this being increased by the expansion of the upper part of the abdomen in deep inspiration. Moritz found in twelve out of thirteen normal persons an increase in intra-gastric pressure proportional to the depth of inspiration. With maximal inspiration he observed that the pressure in the stomach rose to 35, in the rectum to 64 mm. Hg. With sustained inspiration this pressure fell slowly owing, he believed, to the gradual relaxation of the tone of the abdominal muscles. Both in normal and deep breathing the pressure fell at the end of inspiration to the base line, not rising again until the beginning of the next inspiration.

[1] This paper formed the subject of a communication at the meeting of the Society on July 1, 1933.

[2] See reference, Keith, A. 1923.

I have ventured to draw attention to this somewhat forgotten subject because the values quoted and those shown in the figure give an indication of the magnitude of the assistance given to the venous return in a healthy man by the abdominal pressure and the changes it undergoes in inspiration. In addition the graphic records given by Moritz [1895] and my own results support the view that expiration, at any rate in so far as the abdominal muscles are concerned, is a passive process of elastic recoil.

Method.

The observations recorded were made some years ago in the course of an investigation of the intra-pleural pressure by an indirect method. Two mercurial manometers were employed registering simultaneously, one the expiratory force (the intra-pulmonary pressure) in an expiratory effort starting from maximal or lesser degrees of inflation of the lungs, the other the corresponding intra-gastric (extra-pulmonary pressure). The reading of the second deducted from that of the first gives a figure which represents that factor in the expiratory pressure which is due to the elastic contraction of the inflated lungs, and which is therefore a measure of the intra-pleural negative pressure. In the investigation referred to the pleural pressure in three healthy adult men was found to be with maximal inspiration from -14 to -16 mm. Hg. (*Note.* Not yet published.) Through the mouthpiece to which the first manometer was attached passed a thin rubber tube 18 in. long, ending in a small rubber bag. External to the mouthpiece this tube was attached to the second manometer. The bag, which had a size of about 2·5 cm. by 1·5, when filled with air, was swallowed partially deflated and a sufficient quantity of air was afterwards injected through a T-piece on the manometer connection. The observations were made about 3 hours after a meal, swallowing being assisted by a small draught of water. Moritz employed a similar method with a somewhat larger bag and a water manometer.

The pressure changes in the intra-gastric pressure accompanying different types of breathing are illustrated in Fig. 1.

Results.

Graph I in the figure shows a record of the two manometers, the upper (*B*) giving the expiratory force, the lower (*A*) the intra-gastric pressure. Before expiring into the mouthpiece the subject filled the lungs (not to the maximal extent). The effort was sustained for 20 sec. with an intra-pulmonary pressure of 43 mm. The corresponding intra-gastric

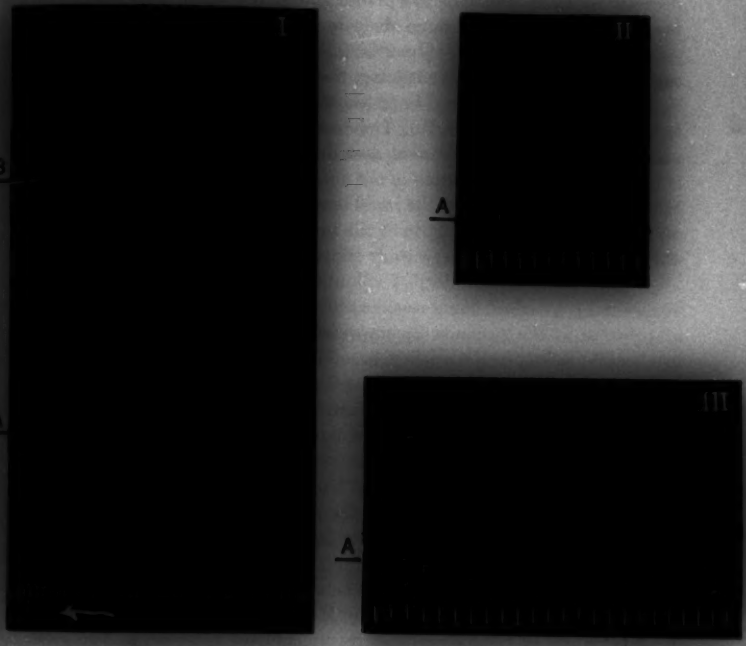

Fig. 1. Respiratory changes in intra-gastric pressure.

I. Subject A. H. Posture: sitting erect. *A*. Intra-gastric pressure: rise of P = 34 mm. Hg. *B*. Intra-pulmonary pressure: rise of P = 43 mm. Hg. Intra-gastric P at base line *A* = 6 mm. Hg. To right of record on base line *A* pressure changes due to voluntary deep breathing. To left of record elevations of pressure accompanying hyperpnœa. Read right to left. Time record 1 sec.

II. Subject D. N. Posture: sitting erect. Voluntary hyperpnœa. Rate of breathing 15 per min. Max. rise of P = 40. *A*. Base line of intra-gastric pressure: P = 5 mm.

III. Same subject and posture. Maximal inspiration followed by maximal voluntary expiration. *A*. Base line of intra-gastric pressure: P = 5 mm. Preliminary normal breathing (to right of record). Inspiration rise of P = 3 mm. Max. inspiration rise of P (3rd curve) = 45 mm. Max. expiration rise of P (1st curve) = 23 mm. Hg. I. Inspiration. E. Expiration. In both II and III breathing was unobstructed through mouth. Read right to left. Time record 2 sec.

pressure being 34 mm. This was preceded by a few fairly deep voluntary breaths and followed by rapid deep breathing, the dyspnœa resulting from the effort and absence of breathing. The effect on the intra-abdominal pressure is seen in the lower tracing, the rises corresponding to inspiration, the falls to expiration. The maximum rise of pressure accompanying the dyspnœa was 24 mm. Hg. The base line represents a pressure of about 6 mm. The fall below this, seen during the dyspnœic hyperpnœa, is probably due to a relaxation of the abdominal muscular tone following the effort or resulting from the rapid breathing. The record of the upper manometer shows no indication of the breathing, as the mouthpiece was only in place during the expiratory effort.

Graph II shows the effect of voluntary deep breathing at a rate of 15 per min., no effort being made to fill the lungs completely. The rise of pressure in the stomach accompanies inspiration (max. 40 mm. in 4th breath) falling to the original base line when inspiration ceases. The intermediate slight rises are due to oscillations of the mercury.

In Graph III the subject was instructed to fill the lungs to their maximal extent, and follow this by a slow maximal expiration.

At the commencement of the record some small pressure changes due to normal breathing occur, a rise of 3 mm. with inspiration, falling to the base line in expiration. During the forced breathing it will be seen that in the third curve the intra-abdominal pressure reached 45 mm. In this case inspiration was maintained for 5 sec., the gradual fall of pressure during that period probably corresponds to the slow relaxation of tone of the abdominal muscles observed by Moritz under similar conditions. With cessation of inspiration the pressure falls to the base line to rise again with forced expiration, 23 mm. being reached in the fourth curve. In this observation, as in that shown in Graph II, breathing was unobstructed.

Discussion.

From the records described above it is evident that, except in Graph III (and to a lesser extent in the first part of Graph I), where expiration is voluntarily carried beyond the normal limit, there is no such rise in the intra-abdominal pressure preceding inspiration such as would correspond to the active participation of the abdominal muscles in expiration. The results are, in fact, exactly those which would be expected if the abdominal walls were composed simply of an elastic material.

A further point of interest in connection with the form of the respiratory cycle appears in these records. It is shown in the pressure changes

accompanying normal breathing in Graph III, but is better seen in Moritz's observations with a water manometer [Moritz (1895), Fig. 5, p. 333], both in normal and deep breathing. Between the first phase of expiration and the succeeding inspiration the graph of intra-gastric pressure shows a horizontal line, indicating that in the cycle of diaphragmatic breathing a pause of some duration occurs forming the second phase of expiration. Gad and Head describe this pause as normal for the rabbit. M. Hammouda and I have described it as being present in the anæsthetized or decerebrate dog. Its occurrence in the normal human cycle has, however, been disputed [Schafer, 1932]. The intra-abdominal pressure changes suggest that the pause is normal in human diaphragmatic breathing.

The influence of the movements of respiration in aiding the return of blood to the heart is of course well recognized. In relation to the figures given, it is of interest to consider briefly the extent of this influence of the intra-abdominal pressure and the changes it undergoes in normal and deep inspiration, combined with the corresponding changes in the intra-thoracic pressure on the return of blood from the vena cava and the organs whose veins open directly into it. The following figures indicate the value of the assistance given in an adult man standing or sitting erect under conditions of (*a*) normal expiration, (*b*) normal inspiration, (*c*) maximal inspiration:

(*a*)	Pelvic P	28 mm. Hg.	Thoracic P	−6	Combined effect	34 mm.
(*b*)	"	32 "	"	−8	"	40 "
(*c*)	"	68 "	"	−15	"	83 "

Converting the combined figures into centimetres of blood, (*a*) represents 43, (*b*) 51, and (*c*) 105. The following measurements may be compared. The average height of the dome of the diaphragm above the pelvic floor is given by Keith as 36 cm. The height of the right auricle (entrance) is about 40 and the highest point of the venous system (vertex) 83 cm. above the pelvic level. Muscular exertion may raise the abdominal pressure 30 mm. or, if the exertion is great (*e.g.* lifting or sustaining a heavy weight), considerably more. The value of the assistance given, against gravity, by the intra-abdominal P to the venous circulation is evident.

The chief abdominal organ affected by these pressure changes is the liver. It is clear that an increase of 45 mm. in the sub-diaphragmatic pressure must, by compressing that organ, greatly assist the outflow of blood into the hepatic veins and vena cava. The subject is possibly of more clinical than purely physiological interest. In conditions in which,

owing to a lack of tone in the abdominal muscles, a very low or negative sub-diaphragmatic pressure exists, it is easy to understand to what an extent the return of blood from the abdominal organs and the vena cava may be hampered.

Summary.

1. There is a rise in intra-abdominal pressure during inspiration proportional to the depth of inspiration.

2. No rise of pressure occurs in normal expiration such as would occur if the abdominal muscles took an active part in that act.

3. In deep breathing the abdominal pressure may exceed the maximum blood-pressure in the vena cava due to gravity.

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612.743

THE ELECTRICAL RESISTANCE OF STIMULATED MUSCLE.

By W. HARTREE.

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The observations recorded here arose from an attempt to verify, or explain, certain anomalous results which appeared in the analyses of the initial heat production of stimulated muscle. In some of these the usual clear division of the initial heat into two phases, corresponding respectively to contraction and relaxation, did not occur, but subsidiary waves or other anomalies were present [see Hartree, 1931]. Earlier experiments were all at 0° C., but further cases of the same kind have recently occurred at room temperature, in the heat analyses to be described in the subsequent paper, using the very rapid Moll microgalvanometer and the quickest thermopile available. It seemed possible that the irregularities found might be due to changes in the thermal contact between muscle and thermopile during the alterations of tension associated with contraction. Moreover, even when the muscle was held as rigidly isometric as possible, small movements would undoubtedly occur, and these might change the flow of heat from muscle to thermopile and so affect the results of the analysis.

A possible test for the accuracy of the analyses, and a means of finding out whether such disturbances did in fact occur, was suggested by Prof. A. V. Hill as follows: Record the thermopile-galvanometer deflection curve (1) on stimulation, (2) on heating by a known amount with a high frequency current producing no stimulus, (3) on stimulating and heating simultaneously. If contraction affected the flow of heat from muscle to thermopile, then the difference between (3) and (1), which is the result of heating by the high-frequency current during contraction, should be different from (2), which is due to heating by the high-frequency current at rest. If the curve obtained by subtracting curve (1) from curve (3) were not the same as curve (2), this could be attributed to the same heating

having a different effect on the thermopile when the muscle was stimulated and when it was at rest, owing to change of shape, change of position, or change in degree of thermal contact.

It appeared immediately that the difference between curve (3) and curve (1) not only differed from curve (2) in shape but was also considerably smaller (about 8 p.c.). The latter result suggested the possibility that the resistance of the muscle was altered by stimulation, and this might be due, at least in part, to a change in the amount of muscle between the electrodes during contraction. It might, however, be caused by a genuine change in the electrical conductivity of the muscle during activity, due to one or other of the physico-chemical changes associated with contraction. The latter possibility was important enough to require further investigation.

The heating current employed was produced by the valve oscillator referred to in a previous paper [Hartree, 1932]; the frequency employed was about 100,000 cycles per second; the strength of the current C was measured by a vacuo-junction supplied by the Cambridge Instrument Company. The hot wire of the vacuo-junction was in series with the muscle: its thermocouple was connected either to the Moll galvanometer or to the moving magnet galvanometer usually employed. On stimulation C^2 was always greater (for the same oscillator volts) by about 7 p.c., and since C^2M (where M is the muscle resistance) was 8 p.c. less, it appeared that the resistance between the electrodes fell about 15 p.c. on stimulation.

A simple way of observing the effect without measuring it was to apply a steady heating current of appropriate strength and, when the deflection of the vacuo-junction had settled, to stimulate the muscle. This resulted in an appreciable increase in the deflection measuring the current in the vacuo-junction, which soon disappeared when the stimulus was over.

It was observed, however, that when the muscle was stimulated, although the ends were fixed as definitely as possible at the bone and the tendon respectively, there was yet a very appreciable motion in the muscle due to the thicker part near the bone extending the thinner part. The actual motion, of the surface at least, can be seen by placing some very small pieces of thread on the muscle and watching them when the muscle is stimulated. The motion of the part several millimetres from the bone end is inappreciable, but that of the thin end, which may extend 6–8 mm. above the top edge of the thermopile, is considerable. This may result in as much as 2 mm. of the muscle passing on to the thermopile during contraction and rather less passing the top electrodes, but quite a

sufficient amount to diminish the resistance between the electrodes considerably.

The muscle, a frog's sartorius (English *R. temp.*), was therefore set up in the reverse position, the bone being at the top and held by the wire to the tension lever. The lower electrode was near the bottom edge of the thermopile, and the tendon held at 2 mm. below this served to avoid having a very thin part of the muscle at this electrode. There was certainly less change than before in the amount of muscle between the electrodes and on the thermopile during contraction. The motion past the top electrode, though some distance from the bone, was inappreciable, and that past the bottom electrode was much less than before, as there was so much less thin muscle beyond the electrode to be extended. Even in this case, however, it is quite appreciable, and readings by thermopile and vacuo-junction taken as before showed an apparent fall of resistance of 7 or 8 p.c., instead of 15 p.c., on stimulation.

Analyses of the initial heat with the muscle in this position are smoother and more regular than previously, which probably shows that there is less motion of the part of the muscle on the thermopile than there was when it was set up in the usual position.

The galvanometer sensitivity was now increased so that the stimulating current only, when passed through the vacuo-junction for 1 sec., produced a good deflection (about 100 mm.), and photographic records of the deflection were made when stimulating the live muscle for 1 sec., when passing the same current through the muscle when dead for 1 sec., and also when passing a constant current through a carbon resistance for 1 sec. The three deflection curves, when reduced to the same maximum, were so similar that there was no doubt that there is little change, if any, of the stimulating current during the stimulus, so there can be little change of the resistance of the muscle during contraction. This method of course will not show if there is a sudden change of resistance immediately on stimulation.

The deflection curve can be analysed to give the current at different times during stimulation, using for the control a curve obtained by passing a current for 1/20 sec. through the vacuo-junction. Such analyses, however, are hardly necessary.

Lastly, a comparatively large steady heating current from the valve oscillator was sent through the muscle and the vacuo-junction, giving a deflection of 1200–1500 mm. The spot of light was brought back to the scale, and a stimulus given with a view to investigating the change of the heating current during stimulation. With the sensitivity used the stimu-

lating current itself, in parallel with the oscillator heating current, had a considerable effect on the vacuo-junction reading. To avoid this it was necessary to stimulate by the nerve, and in order to eliminate as far as possible any effect due to the motion of the muscle past the electrodes, Prof. A. V. Hill kindly supplied an insulated frame with two rigid silver electrodes suitably placed, to which the muscle could be firmly tied. This frame was equipped with a pair of stimulating electrodes, on which the nerve to the sartorius could be laid.

Ten experiments were made with this arrangement, and the results were quite conclusive in showing that when movement is prohibited as far as possible there is no measurable change in the electrical resistance of the muscle during contraction. The extra deflection on stimulation for 1 sec. varied between -10 and $+9$, the mean being about -1. The total deflection was about 1400, so that the electrical resistance was unaltered, on the average to within about 1 part in 1000 during contraction. All results to the contrary are presumably due to movement, change of shape, or similar disturbing factors.

Even when the muscle was tied firmly to the electrodes and there could be little or no motion past them, it was obvious that there was still a change of shape of the part of the muscle between the electrodes, due to the extension of the thinner end by the contraction of the thicker. The maximum motion observed was about 1 mm. Such change of shape must necessarily result in a slight increase of resistance, which no doubt accounts for the negative change (*i.e.* diminished heating) observed in some cases. As regards the positive results, it could be seen that the motion of the muscle past the electrode near the thicker end was very small, even when untied: the motion, however, past the electrode near the thinner end was always in the direction of the thicker end and might be quite large if the muscle were not tied there: thus the effect of any motion past the electrodes due to insecure tying up (and it is impossible to tie very tightly owing to danger of injury) is such as to increase the amount of muscle between the electrodes, and so to diminish its resistance and increase the heating.

Since the maximum effect of contraction on the resistance is less than 1 p.c., and since the mean is less than errors of observation, it seems clear that there is no real change of resistance during contraction.

It is not easy to reconcile the above results with two papers by McClendon [1912, 1927]. In his second paper McClendon found that the impedance of a muscle to a current of 1000 cycles was very much greater when the muscle was at rest than during stimulation. The method

was to place the muscle between two platinum plates and to determine the conductivity (*a*) when the current passing between the plates was too small to excite, and (*b*) when it was large enough to produce contraction. It is possible that the difference between the present results and McClendon's is due to the fact that he employed an alternating current of relatively low frequency, so that the impedance he measured may have been due in part to the impermeability of membranes. With the high-frequency current employed in the present experiments the membranes can have produced very little effect. It is not safe, however, to assume that conductivity is the same with a large current as with a small one, and this and other reasons may have led to McClendon's result. It is impossible, in view of the present results, to suppose that any considerable change in the real conductivity of the muscle can have occurred during contraction.

SUMMARY.

When the movement of a muscle past the electrodes is prevented, its electrical resistance to a current of 100,000 cycles per sec. is not measurably affected by stimulation. The physico-chemical changes, therefore, during contraction are not such as to produce a measurable increase in the number of ions free in the muscle fluid, or in their mobility.

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612.745.3

A REVISED ANALYSIS OF THE INITIAL HEAT PRODUCTION OF MUSCLE.

By W. HARTREE.

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When a frog's sartorius muscle is mounted on a thermopile in the usual way, with a considerable length of the narrow part of it beyond the end of the thermopile, it can be seen on stimulation that there is an appreciable motion of muscle on to the thermopile due to the extension of the thinner part by the contraction of the thicker. This may happen although the ends of the muscle may be quite rigidly fixed. It is possible that this movement may have affected previous heat analyses to some extent, so revised analyses have been made, mounting the muscle in the reverse direction. The lower electrode is placed quite close to the end of the thermopile, and with the new arrangement the thinner end of the muscle is taken only 1 or 2 mm. beyond this electrode and is held firmly by the tendon and tied round fairly tightly at the electrode. Thus, during contraction, the motion of muscle on to the thermopile at this end is very small. At the other end of the thermopile the motion of the muscle on stimulation is negligible. Thus, with this method of mounting the muscle there can be very little change during contraction in the amount of muscle on the thermopile: its shape certainly alters perceptibly but this is unavoidable, and it can only be hoped that this change in shape is not sufficient to have such an effect on the flow of heat from muscle to thermopile as to upset analyses made by means of control heating curves taken when the muscle is in its resting shape.

Results.

A. *Twitches.*

The results of the analyses in several experiments on single twitches at 0° C. were at least fairly uniform. Two curves are given in Figs. 1 and 2, these being somewhat similar to Fig. 5 in a previous paper [Hartree, 1931]. Since no cases like the anomalous figures with multiple "humps," or drawn out initial outbursts (Figs. 6 and 7 in that paper), were found,

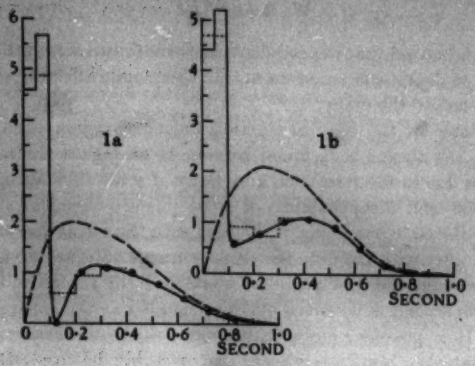

Fig. 1.

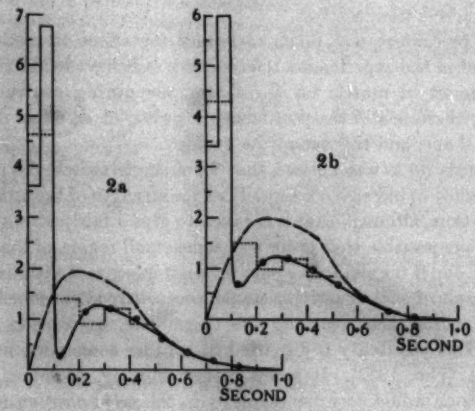

Fig. 2.

Figs. 1, 2. Single isometric twitches of frog's sartorius (English *R. temp.*) at 0° C. Full lines: results of heat analyses using control with 0·05 sec. heating and starting with two steps of 0·05 sec. Dotted lines: results of analyses of the same curves using control with 0·1 sec. heating (for comparison see text). Broken lines for tension curves: these show in every case a distinct "shoulder" between 0·4 and 0·45 sec., presumably due to friction between muscles and upper electrode. The tension curve ends very closely with the heat curve, between 1·0 and 1·2 sec., but cannot be shown clearly beyond 1 sec. Fig. 1 refers to one pair of muscles and Fig. 2 to another pair, these two experiments being chosen to show the greatest observed difference before 0·1 sec. Cases *a* refer to early stimuli and cases *b* to later and somewhat stronger stimuli.

The unit for the vertical scale is the total initial heat per sec. for each case.

it must be admitted that the peculiarities there shown were probably due to motion of the muscle on, or on to, the thermopile affecting the flow of heat from one to the other.

The course of the heat production during a twitch occurs in two phases, as has always been found before. If we regard the mechanical response as due to the formation and decay of some substance, or to the development and disappearance of some physico-chemical state, it is natural to associate one phase of the heat with the formation or development, the other with the decay or disappearance. Regarding the matter in another way, the first phase is waste heat in the development of tension, the second is potential energy degraded as the contraction disappears. It is possible, however (though see the discussion of Fig. 3 below), that the "dip" at 0·1 sec. may possibly be due either (*a*) to motion of the muscle during the rise of tension, or (*b*) to part of the muscle being less active than the rest.

Every precaution was taken to reduce the effect of motion of the muscle, and in the experiments referred to it is believed that the change in the amount of muscle on the thermopile during contraction was negligible; there is still the possibility, however, of an effect due to the change of shape, and this cannot be avoided.

As regards (*b*) it was noticed that for a single twitch the maximum tension seemed to increase continually as the strength of the stimulus was increased; thus, although care was taken to give a fairly strong stimulus it was always possible that there was some small region of inactivity in the muscle, and if the less active part were not in contact with the thermopile the result of such inactivity would necessarily show as a fall in the heat rate, *i.e.* of heat reaching the thermopile, quite soon after the stimulus. This possibility is discussed in another connection in a recent paper [Hartree, 1932]. It seems rather unlikely, however, that the inactive region would occur so regularly in the same position relative to the thermopile as always to give an artificial appearance of a separation between the contraction and relaxation phases of the heat production: though it must be admitted that the same side of the dissected muscle was always outside.

Without further improvement in technique, however, by which change of shape is altogether prohibited, or its possible effect annulled, it does not seem possible to obtain more decisive results.

A few details of the analyses should be mentioned. In every case control heating curves were taken by passing a high-frequency current through the living muscle for 0·05 sec.: this did not stimulate it. When, as in Fig. 2, the heat produced during the first 0·1 sec. is far from

uniform, it is necessary, in order to avoid an early large remainder in the analysis, to start with two steps of 0·05 sec., but there is no advantage in continuing with steps of less than 0·1 sec. From the above control it is easy to construct one for 0·1 sec. heating, and the result of using this is shown by the dotted line; it is clear that the use of such a control in cases in which the heat rate is varying rapidly does not give a good estimate of the heat occurring in each 0·1 sec., and the estimate of that appearing between 0·1 and 0·2 sec. is so wrong as to be very deceptive.

It is obvious that, when using the 0·1 sec. control, any heat really occurring at 0·1 sec. will be considered as half from 0 to 0·1 and half from 0·1 to 0·2 sec. Similarly any heat really occurring uniformly between 0·05 and 0·1 sec. will be considered as ¼ from 0 to 0·1 and ¼ from 0·1 to 0·2. In Fig. 2 there is large heat from 0·05 to 0·1 sec. (not necessarily uniform), consequently the analysis by 0·1 sec. heating control will necessarily show about a quarter of this between 0·1 and 0·2, although in fact there may be no real heat in that interval. A further necessary consequence of taking too much heat in the interval 0·1 to 0·2 is that (to keep the remainders small) too little must be taken in the next step 0·2 to 0·3 sec., etc., the dotted line "oscillating" on each side of the more correct full line, the results being indistinguishable after 0·4 sec. It happens that the dotted lines avoid the "dip" at 0·1 sec. and so perhaps give a more reasonable looking result than the full line, but there is no doubt that the full line gives the best result possible from the observations.

The records for the twitch of Fig. 2a and the corresponding heating control are given for comparison with the cases which follow.

Sec.	0	·05	0·1		0·2		0·3		0·4		0·5		0·6		0·7		0·8		
0·05 sec. control	0	5	50	152	289	434	564	680	776	848	901	939	963	980	991	995	998	999	
Fig. 2a	0		10		106		263		428		578		709		813		890		
Heat	0·180	·34	0·03		0·115		0·12		0·10		0·06		0·035		0·022				
Sec.	0·9		1·0		1·1		1·2		1·3		1·4		1·5		1·6				
0·05 sec. control	1000	999	998	997	995	994	992	990	988	986	984	982	980	978	976				
Fig. 2a	943		975		988		996		999		1000		999		998				
Heat	0·01 approx.																		

Details of the thermopile are given in the appendix. A Downing moving magnet galvanometer was used, sensitivity 1 mm. at 1 m. $= 6{\cdot}5 \times 10^{-9}$ amp., critically damped, total period undamped 0·81 sec., resistance 12½ ohms.

B. *Tetanus.*

A number of experiments were made using the Moll microgalvanometer constructed by Kipp. Although this galvanometer is not sufficiently sensitive to give good sized records for a single twitch, it can be used with excellent results for a tetanus. At 0° C. a tetanus of 0·5 to 1·0 sec. would give a deflection of about 50 mm., and at room temperature a tetanus of 0·2 to 0·3 sec. a deflection of about 100 mm. The mirror of the galvanometer is comparatively large, so good photographic records can be taken at a distance of 4 m. and the spot is always very steady. The action of the galvanometer is so rapid (total period 0·193 sec.), and the thermopile is so quick, that definite analyses can be made using steps of only 0·05 sec. This is necessary for experiments at room temperature (see below), but the galvanometer was also used for a few cases at 0°C., the

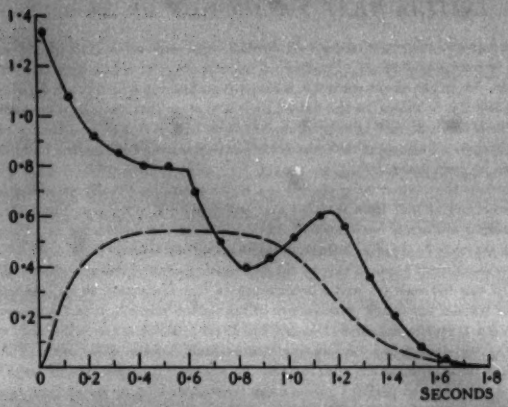

Fig. 3. Full line: result of heat analysis for 0·6 sec. tetanus at 0° C., employing the Moll galvanometer. Broken line for tension. The curves appear to end together. The numbers on the vertical scale must be divided by 1·02 to express heat rates in terms of the total initial heat per second.

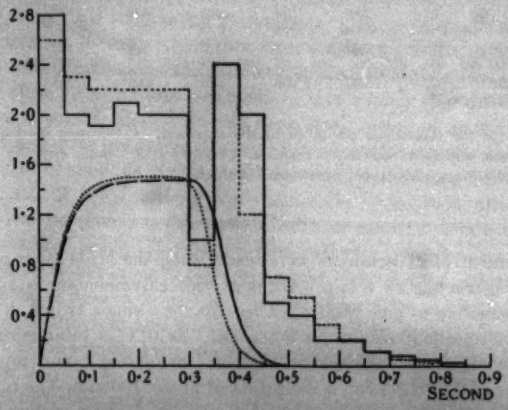

Fig. 4. Results of heat analysis for stimulus of 0·3 sec. approximately, at 17° C. Full line: result of heat analysis using Moll galvanometer and steps of 0·05 sec. Broken line for tension. Dotted lines for an experiment on another day, with stimulus about 0·28 sec., showing very similar results. In each case the heat production lasts much longer than the tension, so that only the early part of the heat production after the stimulus can be associated with the fall of tension energy. The vertical scale gives the heat rate in terms of the total initial heat per second.

result being always very "smooth," as in Fig. 3, of which the observed curves were as follows:

Sec.	0	0·1	0·2	0·3	0·4	0·5	0·6	0·7	0·8	0·9
0·05 sec. control	0	320	833	952	981	993	999	1000	999	998
0·6 sec. stimulus	0	16	73	125	169	213	253	292	324	349
Heat ...	0·067	0·054	0·046	0·043	0·040	0·040	0·035	0·025	0·020	0·022
Sec.	1·0	1·1	1·2	1·3	1·4	1·5	1·6	1·7	1·8	
0·05 sec. control	995	991	987	983	978	973	968	963	957	
0·6 sec. stimulus	372	393	421	448	472	488	496	499	500	
Heat ...	0·026	0·030	0·028	0·018	0·010	0·004	0·002			

Details of the thermopile are given in the Appendix. The Moll micro-galvanometer had a sensitivity of 1 mm. at 1 m. $= 4 \times 10^{-8}$ amp.; it was critically damped and had a resistance of 21 ohms.

It is clear, in this case at least, that the sudden drop in the rate of heat production at the end of a stimulus cannot be due to motion of the muscle, since in Fig. 3 the tension did not begin to fall appreciably for some time afterwards; indeed the rate of heat production had reached a minimum before any movement could have occurred. This is pertinent to the question, discussed above for the case of single twitches, of whether the break in the heat production just after 0·1 sec. might be due to movement.

In this, as in all the following cases, the muscle was mounted in the "reverse" position as described for the case of a single twitch. Usually, after the records had been taken, the muscle was observed when stimulated; its motion past the top end of the thermopile was always negligible and that at the bottom end of the thermopile was usually of the order of 0·5 mm.

The analyses at room temperature (16°–17° C.) agreed well. Several different experiments with stimulus 0·3 sec. gave very similar results, of which two are shown together in Fig. 4. The observed curves for stimulus and control, in the case of the experiments corresponding to the full line, were:

Sec.	0	0·05	0·1	0·15	0·2	0·25	0·3	0·35	0·4	0·45
0·05 sec. control	0	60	359	670	838	914	946	964	976	985
0·3 sec. stimulus	0	8	57	136	224	320	417	512	595	683
Heat ...	0·14	0·10	0·095	0·105	0·10	0·10	0·05	0·12	0·10	0·025
Sec.	0·5	0·55	0·6	0·65	0·7	0·8	0·9	1·0	1·1	1·2
0·05 sec. control	991	995	997	999	1000	1000	999	998	996	993
0·3 sec. stimulus	778	849	896	927	946	973	988	994	998	1000
Heat ...	0·02	0·01	0·01	0·006	0·005	0·004	0·003			

Thermopile and Moll galvanometer as before.

In every case the heat went on very appreciably after the tension had fallen to zero, which must be due to genuine delayed heat, as described

in a recent paper [Hartree, 1932]. It cannot be attributed to delayed appearance of heat, due to inactivity of some of the muscle, as it comes too late for that and is about the same in every case. The early part of the heat production was very regular, in fact it is remarkable how steady was the heat rate during stimulation after the first 0·05 sec.

The analyses with steps of 0·05 sec. were as good and as definite as in the previous case of a single twitch with steps of 0·1 sec. By comparing the tables above it can be seen that the 0·05 sec. control with the Moll galvanometer is now higher at 0·1 sec. than it was with the slower moving magnet galvanometer at 0·2 sec. The analysis would be even better using a control employing still shorter heating, but with the high frequency oscillator available the amount of heat, and therefore the shortness of the heating, is limited. The improvement is entirely due to the quicker galvanometer action since the same thermopile was used in every case.

The Downing moving-magnet galvanometer could not take advantage of the comparatively large amount of heat in the tetanus at a higher temperature since, if it were made very insensitive and very rapid, the damping could not be increased sufficiently. It was necessary, therefore, to increase the damping as much as possible and then to diminish the sensitivity with a resistance.

Summary.

It seemed possible that the analysis of the heat production during the initial phase of muscular contraction might be influenced by slight movements or changes of shape of the muscle during contraction. A modified arrangement has made it possible to eliminate to a considerable degree the effect of such possible disturbances. When this is done the anomalous cases described in an earlier paper disappear and the heat production in a single twitch is found to occur in two phases only—contraction and relaxation. In a tetanus the same two phases occur, and so long as the stimulus is continued the rate of heat production persists at a nearly constant but slightly decreasing rate, following a more or less sharp fall soon after the beginning of the stimulus.

I am greatly indebted to Prof. A. V. Hill for his encouragement and advice.

The Moll microgalvanometer referred to in this paper was purchased with a grant from the Royal Society.

References.

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APPENDIX.

The properties of the thermopile employed.

The instrumental factors involved in the attainment of the greatest possible sharpness of analysis have been discussed by A. V. Hill (*Adventures in Biophysics*, Philadelphia, 1931, Appendix I, p. 142).

The properties of the galvanometers used in the present investigation have been given in the text: those of the thermopile are as follows (a single instrument, constructed by Mr A. C. Downing, was used throughout):

67 flat constantan-iron couples in a distance of 14 mm., resistance 23·7 ohms, bakelite insulation.

Quickness of response. This was determined by analysing a 0·05 sec. heating record by means of the galvanometer deflection due to an impulsive current. The latter might have been obtained experimentally: it was simpler and as accurate, however, to calculate it from the equation

$$y = \alpha t e^{-\alpha t},$$

where $\alpha = 2\pi/T$, T being the complete period (= 0·193 sec.). With this the current in the thermopile, during and after 0·05 sec. heating, had the following relative values:

Sec. ...	0·025	0·05	0·075	0·10	0·125	0·150	0·175
Current	19	45	68	83	89	93	95
Sec. ...	0·200	0·225	0·250	0·275	0·300	0·325	
Current	97	98	99	99½	100	100	

For instantaneous heating half the maximum thermopile current would be developed in about 0·04 sec., 95 p.c. of it in about 0·14 sec. The Moll galvanometer attains 95 p.c. of its full deflection to a constant current in about 0·10 sec., so is slightly faster than the thermopile.

612.816.7

THE EFFECT OF IONS ON THE CUTANEOUS SENSORY ENDINGS OF THE FROG.

By M. TALAAT.

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The effect of various ions on the response of sensory endings has been studied by Matthews [1931] in the case of the frog's muscle spindle. The results agreed with the known effects of ions on excitable tissues, for absence of $Ca^{\cdot\cdot}$ was found to increase the excitability and prolong the discharge of nerve impulses and excess of $K^{\cdot}$ to depress it.

Feng [1933] has shown the depressant effect of increased $K^{\cdot}$ on the sensory endings in the skin, but there has been no systematic work on the behaviour of cutaneous endings in solutions with diminished $Ca^{\cdot\cdot}$ content. The point seemed worth investigating because the cutaneous endings in the frog are mainly of the type which becomes very rapidly adapted to a constant stimulus, whereas muscle spindles adapt slowly. Adrian and Gelfan's recent work [1933] on muscle fibres has emphasized the common basis of rhythmic impulse discharges, and it was hoped that further study of sensory endings might also be of value from this point of view.

Method.

An isolated piece of frog's skin with its nerve supply formed the material for most of the experiments. The skin was usually slightly stretched and pinned at the edges to the floor of a small wax chamber that held about 2 c.c. of fluid. In a few experiments the antidromic preparation devised by Adrian, Cattell and Hoagland [1931] was used. The cutaneous nerve was placed on two silver silver-chloride electrodes, and the nerve endings were stimulated by touching the skin with a glass rod or by application of a 5 g. weight.

The action currents from the nerve were photographed with a Matthews' oscillograph and viewed in a revolving mirror. Observation of the discharge by a loud-speaker system has proved of great value in many of the lengthy experiments.

The chief solutions used were:

		p.c.		p.c.
(1) Ringer	NaCl	0·65	$NaHCO_3$	0·015
	KCl	0·02	$CaCl_2$	0·025
(2) Ca and K free Ringer	NaCl	0·695	$NaHCO_3$	0·015
(3) Ca free Ringer	NaCl	0·675	$NaHCO_3$	0·015
	KCl	0·02		
(4) K free Ringer	NaCl	0·67	$NaHCO_3$	0·015
	$CaCl_2$	0·025		

The osmotic pressures of these solutions differ very little and the anion content is practically the same. As was shown by Matthews [1931], any difference in the behaviour of the end organs when bathed with such solutions must be attributed to the metallic ions.

Other solutions used will be referred to later.

Results.

Absence of Ca *and* K. When the isolated skin preparation is kept in Ringer at room temperature (12–19° C.) no resting discharge can be detected in its nerve supply, unless the area supplied by the nerve has been cut through during the dissection when slow impulses of small magnitude appear. [Cf. Adrian, 1931.]

If the Ringer is replaced by a K and Ca free solution, it is found that, within 5 min., the end organ gives for the same stimulus (a 5 g. weight) a greater discharge than in Ringer (Fig. 1). The frequency of the impulses is increased at the moment when the weight touches the skin, and the discharge continues for a few seconds instead of subsiding almost at once when the weight comes to rest on the skin. This effect increases steadily as the skin remains in the solution, the discharge subsiding more and more slowly under a constant stimulus.

A stage is reached at which the discharge resembles that of a muscle spindle in that it is maintained until the moment when the stimulus is removed. This is followed (20–30 min. after the application of the solution) by a stage in which the discharge outlasts the stimulus. At first the after-discharge is of low frequency and lasts only for several seconds, but after the skin has been in the solution for about 1½ hours it lasts from 3 to 10 min. and is of high frequency. By touching the skin with a fine pointed glass rod it is possible to arouse the activity of single end organs and to study the evolution of this discharge. It begins with a frequency above 100 per sec. and is at first regular (Fig. 2 A). The frequency declines steadily (Fig. 2 B) and the discharge becomes quite irregular when it has fallen below about 25 per sec. But in many

Fig. 1. Sensory impulses in dorsal cutaneous nerve of frog. The effect of dropping a load of 5 gm. on the skin while (A) in Ringer, and (B) 1 min. after application of NaCl, $NaHCO_3$ solution. In both cases the load was left till the end of the record. Time marks (white stripe in B) = 0·6 sec.

Fig. 2. After-discharge developed in NaCl, $NaHCO_3$ solution, skin touched with a fine pointed glass rod. (A) 30 sec., and (B) 60 sec. after removal of the stimulus. (C and D) rhythmic grouping occurring in another ending 3 min. after the stimulus (touch). All records at same speed.

preparations the decline in frequency is associated with a well-marked grouping of the impulses, of the same type as that found by Adrian [1930] in injured mammalian nerves and by Matthews in the frog's muscle spindle in NaCl [1931]. The grouping is shown in Fig. 2 C, D. The groups usually develop as they do in mammalian nerves. The number of impulses in the group increases gradually from two to five or more and, as the number increases, the interval between the first impulses in the group becomes shorter and shorter, so that eventually the second impulse may fall within the relative refractory period of the first [cf. Adrian, 1930, Figs. 4, 5]. As in mammalian nerves a definite increase in the number in the group is preceded by a stage in which the number fluctuates between the lower and the higher value.

In addition to this increased activity on stimulation, some of the end organs begin to show a resting discharge, but usually this is of low frequency and is irregular.

The preparation recovers readily if put again in Ringer's solution.

Absence of Ca. When the fluid contains 0·02 p.c. KCl in addition to the NaCl the discharge becomes prolonged in the same way but the frequencies are lower than in NaCl alone. The discharge which occurs as the immediate response to a stimulus is, if anything, slightly less than in ordinary Ringer. The slowing of adaptation is shown by the development of a resting discharge (the skin being under slight tension) and usually a stage is reached at which a touch gives a long after-discharge like that in NaCl. The maximum frequency (60 per sec.) is not so high; grouping of the impulses may occur but the spacing of the impulses in the group is wider than in NaCl (30–50 per sec.).

The absence of Ca does not seem to be detrimental to these end organs, as they are still excitable after a period of 5 or 6 hours in this solution.

Absence of K. When the fluid contains Ca, but not K, the response of the end organ does not differ appreciably from that in Ringer except that a slow resting discharge may appear. No after-discharge was noticed in any of the eleven experiments made.

Absence of electrolytes. It may be mentioned that, if the skin is immersed in isotonic cane-sugar solution, the excitability of the end organs disappears in about 1–1½ hours. On replacing the sugar by the NaCl, $NaHCO_3$ solution the excitability returns to some of the end organs within a minute and to practically all of them within 10–15 min. This suppression of activity and recovery can be repeated several times without apparent harm to the end organs.

Solutions containing oxalate or citrate. Since the factor which causes the prolonged discharge is the absence of Ca ions, it is to be expected

that solutions of sodium oxalate or citrate would produce a much greater effect than NaCl alone. This is true for the stimulation of muscle fibres and Loeb [1905] has shown that application of an oxalate solution to the skin of a frog with intact spinal cord produces great reflex activity.

The first experiments were made with 0·6 p.c. Na oxalate solution. As this is hypotonic and contains no K it was replaced later by a solution made up of eight parts of Ca free Ringer and two parts of 1·2 p.c. Na oxalate, but as far as could be observed the results were the same.

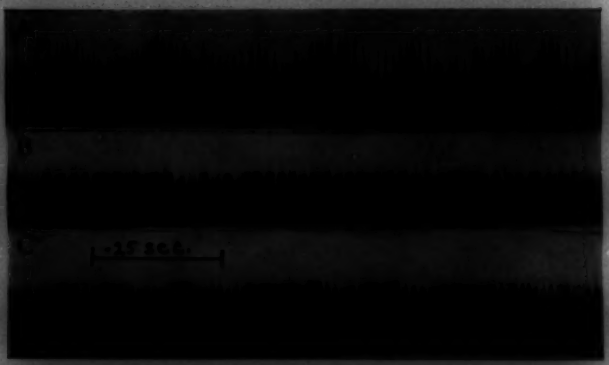

Fig. 3. Resting discharge (A) in 0·6 p.c. Na citrate (B and C) during recovery after isotonic oxalated solution. (B) 30 min., and (C) 60 min. in Ringer. All records at same speed.

The main result is a development of continuous activity in all the sense organs. The first noticeable effect is an increase in the response to a test stimulus (*e.g.* 5 g. wt. or light touch) and then a resting discharge develops (the skin being under slight tension). It appears first in nerve fibres giving small impulses conducted slowly, but soon the larger impulses appear and within 30–40 min. all the sensory endings in the skin seem to be discharging at a high frequency. The continuous activity lasts for about 5 or 6 hours and the frequency is apparently unchanged. Some of the records (Fig. 3 A) give the impression that the impulses in several fibres are discharged synchronously, as they may be in damaged nerve or muscle [Adrian, 1930; Adrian and Gelfan, 1933]. At the end of 5 or 6 hours the end organs seem to fail, one after the other,

and when all traces of the discharge have disappeared the skin is found to be inexcitable to any form of stimulation.

If Ringer is substituted for the oxalate solution before this failure has occurred the discharge subsides gradually but some of the end organs may still be discharging after 30 min. in Ringer (Fig. 3 B). Towards the end a single end organ may remain in action, but its activity is often intermittent (Fig. 3 C).

A few experiments have been made with the antidromic skin preparation in order to analyse the discharge in the individual nerve fibres. In this preparation it was found that the effect took longer to develop and lasted for about 1 hour only, unless the skin area giving the antidromic impulses was repeatedly irrigated with the oxalate solution. This may well be due to the skin having an intact blood supply.

With this preparation the frequency of the discharge in single nerve fibres was found to be as a rule between ten and forty per sec. It remains practically unchanged so long as the end organ is active. It is irregular below twenty to twenty-five per sec. but fairly regular above this. The frequency can be increased by touching or stretching the skin, and once a higher frequency is obtained it persists for a long time although the skin is allowed to relax completely.

The same results have been obtained with solutions of sodium citrate instead of oxalate.

Discussion.

The action of the oxalate solution probably differs from that of the NaCl solution only in so far as it secures a more complete removal of Ca from the skin. Thus a sensory ending of the rapidly adapting type shows the following changes when the $Ca^{"}$ ion concentration is progressively lowered. At first the discharge to a prolonged stimulus, instead of declining rapidly, persists for a greater and greater time. A stage in which the discharge coincides with the stimulus is soon followed by one in which there is a more and more prolonged after-discharge, and eventually a touch is enough to produce a discharge of high frequency lasting for 10 min. or more. At this stage the removal of the stimulus has little or no effect on the activity of the ending. As far as the stimulus is concerned the excitation is not now a reversible process, though the activity declines gradually and ceases sooner or later.

This stage recalls the action of NaCl solution on muscle fibres, for Adrian and Gelfan found that before spontaneous activity appears,

there is a period in which touching or stretching the fibres gives a discharge which may cease when the stimulus is removed but often outlasts it for a variable time. They pointed out that this behaviour suggested that of a sensory ending in which the mechanism of excitation had become brittle and inflexible instead of responding smoothly to the rise and fall of the stimulus.

Thus the three classes of excitable structure, muscle fibre, rapidly adapting and slowly adapting sense organ can be arranged in order with regard to mechanical stimulation and to the effects of diminished calcium. Normally a muscle fibre does not respond at all unless the stimulus is of damaging intensity; in NaCl it may give an abrupt, ungraded response which often outlasts the stimulus, and eventually prolonged discharges appear without any stimulation.

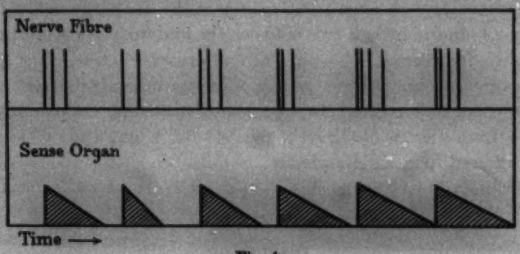

Fig. 4.

The rapidly adapting end organ responds normally by a brief discharge which declines at once although the stimulus is continued. In sodium chloride the adaptation becomes less and less rapid and ultimately the discharge, once it has been started by the stimulus, continues for a time which seems to depend only on the general state of the ending. At this stage there is not much difference between the response of the muscle fibre and of the nerve ending, but the latter preserves a little of its specialized properties, for its discharge can still be increased in frequency by an increase in the stimulus. A slowly adapting end organ such as a muscle spindle shows the most flexible discharge mechanism, for it combines a slow rate of adaptation with an excitatory process which follows every variation in the stimulus. It needs no reduction in Ca ions to enable it to give a prolonged discharge, and the discharge ceases at once when the stimulus is withdrawn.

The present records show one other point of interest, the development of the grouped discharge in the absence of Ca. The slow rate of adaptation seems to be associated with the tendency for each pulse of activity in the end organ to discharge not one but several impulses down the nerve fibre. The impulses are usually grouped as they would be if the pulse of activity which arouses them starts at its maximum intensity and declines gradually. Thus the lack of Ca prolongs the duration of the whole discharge and also prolongs the effect of each active period in the end organ. The relation between nerve ending and nerve fibre activity may be represented as in Fig. 4. The diagram recalls the slow potential waves found by Adrian and Gelfan in muscle fibre at the region of origin of a rhythmic discharge. In that case the waves rise slowly, but a series of potential waves rising steeply and declining slowly would account very well for the grouped discharges in the present case.

Summary.

When the frog's skin is treated with solutions containing $Na^{\cdot}$, or $Na^{\cdot}$ and $K^{\cdot}$, but no $Ca^{\cdot\cdot}$, there is a much less rapid adaptation of the sensory endings to a prolonged stimulus. Eventually the discharge, once started, may continue long after the stimulus has ceased. These changes develop sooner in solutions containing oxalate and the endings may remain in continuous activity for 5 or 6 hours. The impulses are often discharged in groups. The results are discussed in relation to the behaviour of muscle fibres and of sense organs of the slowly adapting type.

I am greatly indebted to Prof. E. D. Adrian for suggesting the work and for help in the preparation of this paper and to Mr B. H. C. Matthews for constant advice.

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THE RESPIRATORY QUOTIENT OF THE EVISCERATE CAT.

By JAMES McINNES PETERSON.

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It is well known that the respiratory quotient of a spinal or decerebrate animal is higher after abdominal evisceration. This raised quotient has been observed in certain instances to attain a value of unity [Porges and Salomon, 1910; Best, Dale, Hoet and Marks, 1926] which has been accepted as added support for the view that the oxidative metabolism of skeletal muscle involves carbohydrate alone. On the other hand, respiratory quotients below unity have been observed for the abdominally eviscerated animal [Markowitz, 1928; Irving and Foster, 1930].

Evidence from other sources is also divided in regard to this question. The work of Meyerhof and his colleagues [1920, 1924] indicates that the quotient for the isolated muscle of the frog is unity, while Takane [1926], from the same laboratory, obtained a mean quotient of 0·79 for strips of rat's diaphragm.

Doisy and Beckmann [1922], Himwich and Castle [1927] and Himwich and Rose [1929], by analysis of arterial and venous blood of the limb muscles, obtained values for the respiratory quotient of mammalian muscle which are approximately those obtained with the intact animal.

It has frequently been suggested that the observed quotient is unreliable as a guide to the nature of the oxidative metabolism of a muscle preparation because of the variations in the CO_2 output which may occur through changes other than those involving oxidation [Verzár, 1911; Murlin, Edelmann and Kramer, 1913; Krogh, 1916; Kilborn, 1928; Kilborn, Soskin and Thomas, 1928; Bornstein, 1929; Ferguson, Irving and Plewes, 1930].

There are two such changes, which are, however, not independent, namely a "blowing off" of CO_2 by over-ventilation, and a displacement of CO_2 from chemical combination in the tissues by accumulating lactic

acid. It is known that lactic acid will cause an increased ventilation, and that over-ventilation will in turn cause an increase in lactic-acid production [Eggleton and Evans, 1930]. The purpose of this paper is to discuss the relationship which has been observed to exist between the changes in lactic acid and CO_2 concentration of the blood and the respiratory quotient.

Methods.

The methods employed throughout were those described by Anderson, etc. [1931]. The respiratory metabolism of the cat, decerebrated under ether and abdominally eviscerated, was measured by the Douglas bag method. Blood lactic acid was estimated by the method of Friedmann, Cotonio and Shaffer, and the blood CO_2 content of samples of blood, kept from contact with air, was determined with the van Slyke manometric apparatus.

Results and discussion.

The closeness of the relationship between the lactic-acid accumulation in the blood and the loss of CO_2 therefrom was shown by Kilborn, Soskin and Thomas [1928], employing the spinal cat. In the present work this relationship has been investigated in some forty experiments, the results of which are typified in Fig. 1 (Exps. 34, 39). Following decerebration, the blood lactic acid may be high (Fig. 2, Exp. 142), but rapidly falls to a minimum value at which it may remain with only slight increase over a number of hours. On evisceration, however, there is an increase in blood lactic acid, which is paralleled by a decrease in CO_2 and a rise in the respiratory quotient. This sudden increase is, however, of short duration and gives place to one which is more gradual or even to a slight or temporary decrease (Exps. 39, 42), the fall being accompanied by an equally small increase in the blood CO_2. When the lactic acid and CO_2 of the blood have reached values of about 80 mg./100 c.c. and 25–30 c.c./100 c.c. respectively, the increase of lactic acid and decrease of CO_2 are suddenly accelerated. This point of change has been termed the "break point."

In order to determine the relationship of these changes to the respiratory metabolism, the figures obtained in six experiments (31, 33, 34, 36, 39 and 41) have been subjected to a careful examination. In Fig. 3 are plotted the points representing all the simultaneous values for lactic acid and blood CO_2 concentrations, and their degree of correlation, r, has been determined by the Karl Pearson formula. The

degree of negative correlation is high, r being $-0{\cdot}93 \pm 0{\cdot}014$. In Fig. 4 all these values have been grouped in sub-ranges of small magnitude (10 mg. of lactic acid and 5 c.c. of CO_2 per 100 c.c. of blood) and the mean of each sub-range plotted. The line drawn through these points,

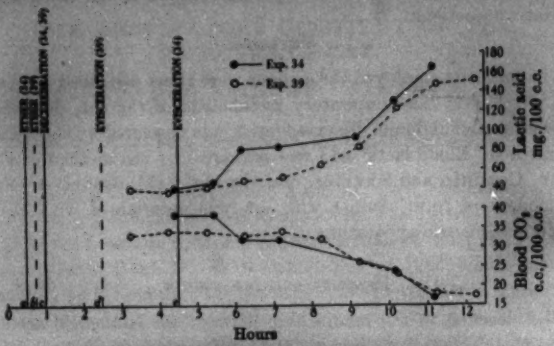

Fig. 1. The relationship of blood lactic acid to blood CO_2 in the decerebrate-eviscerate cat.

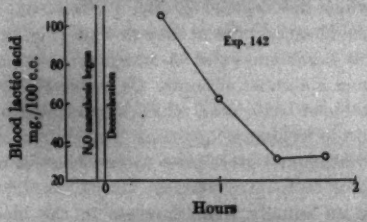

Fig. 2. Change in blood lactic acid concentration immediately following decerebration.

in Fig. 4, is not an attempt to apply the best-fitting straight line, but is an expression of the relationship between the quantities of lactic acid neutralized and the volume of CO_2 displaced in the simple double decomposition reaction:

$$NaHCO_3 + CH_3.CHOH.COOH \rightarrow CH_3.CHOH.COONa + H_2O + CO_2.$$

It is apparent that 1 g. molecule (90 g.) of lactic acid will displace 22·4 litres of CO_2 at S.T.P., or 90 mg. will displace 22·4 c.c.

Whether or not the CO_2 is displaced from the blood by this reaction, it is evident that the two changes, lactate increase and CO_2 decrease, progress at rates which have a stoichiometric relationship, and it is to

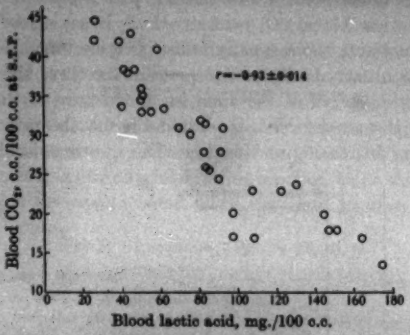

Fig. 3. The values of lactic acid and CO_2 of the blood observed simultaneously at frequent intervals in six experiments.

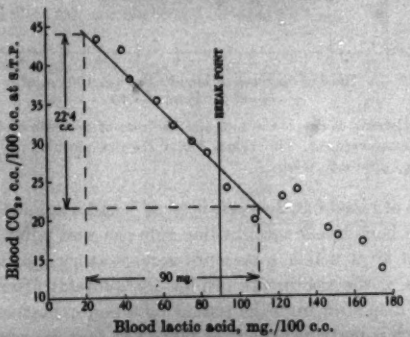

Fig. 4. The circles represent the concurrent changes in the concentrations of lactic acid and CO_2 of the blood. The straight line represents the quantitative relationship between the lactate and displaced CO_2 in the reaction

$$NaHCO_3 + CH_3.CHOH.COOH \rightarrow CH_3.CHOH.COONa + H_2O + CO_2.$$

be expected that the observed respiratory quotient will be higher when the rate of fall of blood CO_2 is greater; an examination of the observed respiratory quotient values for these six experiments shows this to be the case. Coincident with the decrease in blood CO_2 on evisceration, is a

rise in the respiratory quotient which, during the subsequent period of slow accumulation of lactic acid, as in Exps. 33 and 39[1], may maintain a fairly constant value, only to rise with the increase in rate of blood CO_2 loss after the "break point."

It is not a low blood CO_2 content which is associated with a high respiratory quotient, but a rapidly falling CO_2 concentration. In Fig. 5 are shown the observed respiratory quotient for these six experiments; each value, irrespective of the time in the history of the experiment during which it was observed, is plotted against the rate at which the blood CO_2 was decreasing at the time. The quotient is obviously high

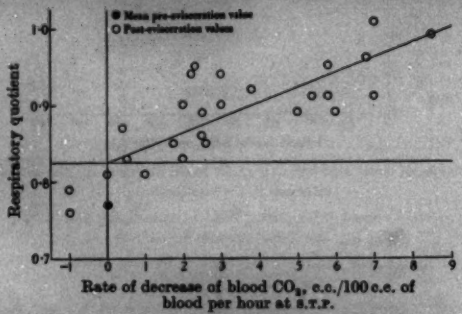

Fig. 5. The relationship between the R.Q. and the rate of decrease of blood CO_2 in the decerebrate-eviscerate cat. Two values found are not plotted: (1) CO_2, 23·0; R.Q., 1·16: (2) CO_2, 12·6; R.Q., 0·98.

when the rate of blood CO_2 decrease is rapid[2], and a splined line approximating to the best-fitting straight line cuts the zero point on the blood CO_2 axis at a level which represents a respiratory quotient of about 0·825. Hence, if the rise in the respiratory quotient after evisceration is due to CO_2 displaced from the tissues by a non-oxidative process, the extent of which is measurable by the blood CO_2 loss, we can assume that the true oxidative quotient of the eviscerate preparation is somewhere

[1] Markowitz [1928] observed that the respiratory quotient of eviscerated dogs was lowered by the injection of glucose. This is in agreement with the finding that in the eviscerate cat the injection of fluid is attended with a delayed accumulation of lactic acid and a low respiratory quotient, probably to be explained as due to the maintenance of a high blood-pressure and a rapid circulation resulting from a lowered blood viscosity.

[2] It is of interest that Himwich and Castle [1927] observed only very slight changes in the CO_2 content of successive blood samples in their experiments in which they found the respiratory quotient of the resting skeletal muscle of the dog to be less than 0·8.

in the region of 0·8. Quotients of this order have been observed in the eviscerate preparation where the blood-pressure was well maintained (as in Exp. 39, in which the animal received an infusion of glucose solution) and the blood CO_2 content was not suffering any significant diminution. Irving and Foster [1930] express their belief that the true respiratory quotient of the spinal eviscerate cat is approximately 0·8. The smallness of the number of values here examined makes it difficult to be precise as to this probable basal value. In Fig. 5, in black, is shown the respiratory quotient for the non-eviscerate preparation, and the one quotient value for the eviscerate preparation which fell below this non-eviscerate quotient is one obtained during a period when the preparation, Exp. 39, had a decreasing blood lactic acid and increasing blood CO_2

TABLE I. Experiments 31, 33, 34, 36, 39, 41.

a. Observed repiratory quotient.
b. Observed CO_2 expired: c.c. at S.T.P. per kg. per hour.
c. Calculated CO_2 output to give, with the observed O_2 consumption, a respiratory quotient of 0·825: c.c. at S.T.P. per kg. per hour.
d. Volume of CO_2 which has to be subtracted from the observed CO_2 output to convert the observed R.Q. to 0·825: c.c. at S.T.P. per kg. per hour.
e. Rate of fall of blood CO_2: c.c. per 100 c.c. of blood per hour.

	a	*b*	*c*	*d*	*e*
Exp. 31.	1·16	296	248	48	23·0
	1·07	291	218	73	7·0
	0·94	262	222	40	3·0
Exp. 33.	0·91	251	229	22	5·4
	0·83	229	228	1	0·5
	0·86	226	218	8	2·5
	0·92	268	240	28	3·8
	0·95	295	256	39	5·8
Exp. 34.	0·87	250	237	13	0·4
	0·85	216	205	11	2·6
	0·96	299	268	31	6·8
Exp. 36.	0·98	255	215	40	12·6
	0·89	236	219	17	2·5
	0·91	157	142	15	7·0
	0·85	163	157	6	1·75
Exp. 39.	0·76	208	220	−12	−1·0
	0·81	—	—	—	0·0
	0·81	252	256	−4	1·0
	0·79	218	228	−10	−1·0
	0·83	—	—	—	2·0
	0·89	237	235	2	6·0
	0·90	310	285	25	3·0
	0·89	389	369	20	5·0
Exp. 41.	0·90	209	192	17	2·0
	0·91	253	224	29	5·8
	0·94	218	191	27	2·2
	0·95	302	262	40	2·3
	0·99	430	358	72	8·5

concentration. If we accept this value of 0·825 as the approximately true respiratory quotient of the eviscerate cat, the extra volume of CO_2 required to raise the respiratory quotient from this basal value to the observed value may be calculated in each instance and correlated with the rate of fall of blood CO_2 (Table I).

A preliminary examination of these values suggested that their relationship could probably be represented by a straight line. The scattered nature of the results made it desirable that the decision on this point, if such were possible, should be made by a qualified statistician and, by the courtesy of Dr J. F. Tocher, who kindly submitted the data to a thorough investigation, I am able to give the following report.

"A preliminary study of the individual observations was made, the means and standard deviations of both series being determined. The two straight lines were then fitted by the method of least squares. It was found that for both straight lines one observation was exceptional and, on examination, this was found to be the first observation. It was decided, therefore, to omit this observation from the series. Two other observations, the second and last, also appeared somewhat exceptional, but were included in the further study.

"The raw data are not suitable for testing linearity owing to the small number of observations in both series. In order, therefore, to carry out the test satisfactorily, the two series were divided into sub-ranges. If x = rate of elimination of CO_2 from blood in c.c. per 100 c.c. of blood per hour, all observations of x from -2 up to and including zero were taken and the mean found. Similarly, the mean value of x was found for the observations above zero up to and including 2 c.c., above 2 c.c. up to and including 4 c.c., and so on, the observations above 8 c.c. being taken together in the last group. Thus we have six sub-ranges or categories of x with definite ranges. The values of y = the volume of CO_2 expired in excess of the basal rate, were classified sub-ranges; these, commencing with the lowest being below -15 up to and including -10, below -10 up to and including -5, etc. Thus the observations were classified in steps of 5 c.c., a grouping warranted owing to the scattered nature of the data. The data were now in a condition for statistical analysis and the observations were recorded in a table of double entry and the mean, standard deviation, correlation coefficient and correlation ratio determined. It was found that the correlation between the two series was fairly high. The mean of the y arrays of x were plotted and the straight line $y_x = \bar{y} + \frac{ry}{x}(x - \bar{x})$ calculated. The observed mean of arrays and the best-fitting straight line are shown on the accompanying diagram (Fig. 6). The valid tests for goodness of fit were applied and it was found, for the small number of observations made, that the theoretical straight line was a reasonable fit to the data. In other words, when sufficiently broad sub-ranges of both series are taken, the volume of CO_2 expired in excess of the basal rate is on the average proportional to the rate of elimination of CO_2 from the blood."

When the probable experimental error is considered, it is apparent that the figures for CO_2 in excess of the basal rate may vary within the limits of 5 c.c. per kg. per hour: the grouping together and averaging of the values within this range is, therefore, desirable and justifiable. Accepting the relationship as linear, it is to be observed, from Fig. 6, that the loss of an additional 1 c.c. of CO_2 from 100 c.c. of arterial blood per hour is accompanied by the addition to the expired air of 4·5 c.c. of CO_2 per kg. of animal weight per hour, over and above that necessary to maintain the respiratory quotient at 0·825. If we assume that the elevation of the respiratory quotient above this value is due to the

CO_2 displaced by lactic acid from the tissues and that the density of the blood is 1·06, these figures show that the equivalence of $\frac{106 \times 4{\cdot}5}{10} = 47{\cdot}7$ p.c. of the body weight of the preparation, including the blood, is losing CO_2 at the same rate as the blood. If this is so, the figures obtained in these experiments offer a complete explanation of the variations in respiratory quotient after evisceration. The weight of the skeletal muscles of the cat according to the figures of Ferguson, Irving and Plewes [1929] amounts, on the average, to 44 p.c. of the total body weight. Higher figures are recorded by Best, Hoet and Marks [1926]. Allowing for the weight of viscera removed, the muscles therefore comprise about 49 p.c. of the weight of the eviscerate preparation, while the blood may be assessed at from 5 to 6 p.c. [Ferguson, Irving and Plewes, 1930]. On the assumption that muscle loses CO_2 at approximately the same rate as the blood, it is possible from these figures to explain the heightened values for the respiratory quotient, but it yet remains to be seen whether this assumption is justified.

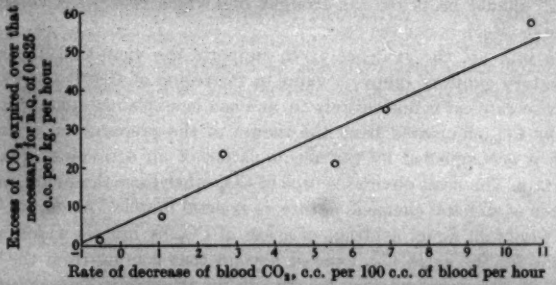

Fig. 6. The relationship of the rate of decrease of blood CO_2 to the excess of CO_2 expired over that necessary (with the observed oxygen retention) to maintain an R.Q. of 0·825.

It is of interest to speculate as to where the lactic acid comes from, and whether or not the CO_2 is lost as a result of the acid accumulation. The evidence so far obtained merely indicates that there is a concurrent equimolecular rate of change in the concentrations of CO_2 and lactate of the blood. The question as to whether either of the two processes, lactic acid increase or CO_2 decrease, can be considered as cause or effect remains open[1]. If we are to assume that more than 40 p.c. of the preparation is losing CO_2 at the same rate as the blood, as the result of a buffering process, the maintenance of a linear relationship between blood lactic

[1] The view that the lactic acid is produced as a result of hyperpnœa and is not its cause can be accepted for the decerebrate preparation only if some explanation of the cause of the hyperpnœa is forthcoming. The possibility of direct stimulation of the respiratory centre by a blood clot has been considered and the brain stems examined after each experiment without any results justifying this explanation. It has been stated that the activity of the respiratory centre is increased after decerebration [Winton and Bayliss, *Human Physiology*, 1930, p. 429].

acid increase and CO_2 decrease must mean that the tissue which shares with the blood the rôle of buffering, must make with the latter a single system, the diffusion of lactic acid between them being rapid. This finding is further borne out by Fig. 5. If we accept the excess CO_2 expired (over that necessary to maintain the respiratory quotient at 0·825) as an index of the part played by $NaHCO_3$ as a buffer, it is apparent that with any variation in the proportionate part played by blood bicarbonate in this process, the relationship between the excess CO_2 and the rate of decrease of blood CO_2 will also vary. That is to say, if the blood plays a proportionately greater part in buffering after the "break point" than before it, values, in Fig. 5, representing conditions after the "break point," should lie below the straight line which represents conditions before it. Such is not the case.

To sum up, the evidence given supports the view that the raised respiratory quotient (above a value in the region of 0·825) observed in the eviscerate cat is due entirely to, and can be explained quantitatively by, the CO_2 eliminated from the tissues of the preparation, a process which is accompanied by the accumulation of an equivalent quantity of lactate. The most obvious source of CO_2 other than the blood, on the grounds of size and chemical nature, is skeletal muscle. Attempts, however, to obtain direct evidence of a loss of CO_2 by muscle which would account for the extra CO_2 expired beyond that coming from the blood have not given confirmatory results. In an attempt to find the source of the CO_2 loss from the over-ventilated cat under amytal, Ferguson, Irving and Plewes [1929] measured the decrease in muscle and blood CO_2, but were unable to find a sufficient diminution. On the other hand, when the animal was ventilated with a gas mixture of high CO_2 tension, it retained a quantity of CO_2 greater than could be accounted for by the increase in the CO_2 content of the muscles and blood. From this it would seem that, although the CO_2 lost during over-ventilation is not traceable directly to a loss from the muscles and blood, the assumption may be made that it is produced by a process that is not necessarily oxidative [Ferguson, Irving and Plewes, 1930]. Irving and Foster [1930], by direct muscle analysis, found losses of CO_2 which were, however, inadequate to account for the high respiratory quotient values observed.

The view that the respiratory quotient of a muscle preparation during its normal metabolism is less than unity, removes what was apparently a distinction between the nature of its metabolism and that of the heart. Starling and Evans [1914] observed that the average

respiratory quotient of the heart-lung preparation was about 0·85. Similar figures have been obtained by Clark and his colleagues [1932] for frog's heart.

From these results it is clear that in the use of the eviscerate mammalian preparation in the study of carbohydrate metabolism, observations of the quotient, in absence of information regarding the rate of loss of CO_2 by direct displacement from the tissues of the body, cannot be relied on in the accurate computation of metabolic changes.

The respiratory quotient of the abdominal viscera.

On the assumption that the removal of the abdominal viscera does not alter qualitatively the oxidative metabolism of the tissues that remain, the observed change in respiratory quotient, from 0·77 to 0·825, must be due to the removal of an oxidative process for which the respiratory quotient is low. This quotient can be readily calculated from the following data:

	Decerebrate cat	Decerebrate eviscerate cat
Oxygen consumption (c.c. per kg. (nett wt.) per hr.)	360·00	245·000
Respiratory quotient	0·77	0·825
Weight (kg.)	2·0	1·800

These values of oxygen consumption are in close agreement with those recorded by Evans, Tsai and Young [1931] for the decapitate cat, viz., 365 c.c. for the non-eviscerated and 250 c.c. for the eviscerated animal. From the figures given, and the assumption already made, it is obvious that the respiratory quotient of the removed viscera is

$$\frac{(360 \times 0{\cdot}77 \times 2) - (245 \times 0{\cdot}825 \times 1{\cdot}8)}{(360 \times 2) - (245 \times 1{\cdot}8)} = 0{\cdot}69.$$

That the low value of this quotient is due to gluconeogenesis in the liver is the ready explanation which has already been discussed by other workers [Evans, Tsai and Young, 1931].

Summary.

In the decerebrate-eviscerate cat, the quantitative relationship between the accumulation of lactate in the blood and the loss of blood CO_2 is shown to be identical with that between lactate and displaced CO_2 in the reaction:

$$NaHCO_3 + CH_3.CHOH.COOH \rightarrow CH_3.CHOH.COONa + H_2O + CO_2.$$

A close relationship is demonstrated to exist between the rate of blood CO_2 loss and the observed respiratory quotient, and evidence is adduced which supports the view that high quotients, obtained with the eviscerate preparation, are elevated above the true quotient by CO_2 displaced from the tissues.

This problem was suggested by Prof. J. J. R. Macleod, whose constant help I gratefully acknowledge. I am indebted also to Dr R. A. Cleghorn and to Dr I. A. Anderson for much assistance in the experimental work.

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The presence in the brain of a substance resembling acetyl choline. By B. B. Dikshit. (*Department of Materia Medica, University of Edinburgh.*)

Extracts of brains of cats and rabbits were prepared by macerating different portions of the brain in an acid-water-acetone mixture (75 c.c. acetone, 10 c.c. *N*/100 acetic acid and 15 c.c. water). The emulsion was allowed to stand for 24 hours at a temperature of 5° C.; it was then filtered and the filtrate was evaporated to dryness *in vacuo* at room temperature. The residue was dissolved in water and this solution was used for injections.

These extracts produced actions similar to those of acetyl choline when tested on the blood-pressure of cats and on isolated rabbit's gut, namely a depressor and an augmentor action respectively. These actions were completely abolished by atropine. The extracts also were rendered inactive by an esterase isolated from horse serum which had been shown to have a specific action of destroying acetyl choline [Stedman *et al.*, 1932].

The quantity of acetyl choline-like substance present varied from 0·5–4·0 γ of acetyl choline per g. of the brain substance. The concentration was greatest in the basal ganglia and least in the cerebellum; the cortical portion had a concentration slightly lower than that present in the basal ganglia.

Acetyl choline injected into the lateral or the third ventricle of cats produced an action different from that produced by intravenous injections. It had only a slight effect on the blood-pressure while the respirations were markedly depressed or completely inhibited. This action was shown to be central and not peripheral by the fact that the movements of strips of diaphragm with all vascular connections severed, but retaining nervous connections through the phrenics, were inhibited by intraventricular injections of acetyl choline.

Stimulation of the central end of the vagus produced an effect very similar to that produced by intraventricular injections of acetyl choline. The action of intraventricular acetyl choline and the effects of stimulation of the central end of the vagus on the respiratory movements were not abolished by atropine given intravenously or intraventricularly.

The cerebro-spinal fluid (cisternal puncture) of 30 cats was tested on the blood-pressure of cats and on isolated ventricular strips from frogs. No acetyl choline-like substance was found in most cases. In two cases an acetyl choline-like action was demonstrated, and this effect was increased in fluid collected after stimulation of the central end of the vagus.

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On the significance of the action of insulin on the R.Q. of the diabetic heart. By E. W. H. Cruickshank. (*Department of Physiology, Dalhousie University, Halifax, N.S., Canada.*) (*Preliminary communication.*)

The belief that fat can be transformed into carbohydrate cannot easily be dissociated from the idea that the primary defect in pancreatic diabetes is one of over-production of sugar.

If in maintaining the over-production theory one postulates that the liver is the site of conversion of fat to carbohydrate, then the removal of the liver would mean a cessation of conversion with a complete failure of fat utilization, leaving carbohydrate, and perhaps protein, as the available sources of energy. This would mean a change in the R.Q. from 0·70 to above 0·80. But the fact that the heart in aglycæmia continues to function most efficiently with a R.Q. maintained at 0·70 [Cruickshank and Startup, 1933] would eliminate the liver as the sole site of conversion, but not the possibility of such a phenomenon.

If we assume that the primary defect in pancreatic diabetes is not a failure in oxidation, that the sole function of insulin is to prevent over-production of sugar from fat in the liver and that the heart cannot be regarded as the site of transformation, then the isolated diabetic heart should always show a R.Q. of unity in the presence of so much available sugar, and the addition of insulin should produce no change in its carbohydrate metabolism.

If carbohydrate oxidation is not impaired and the primary defect is

over-production and if it be maintained that insulin inhibits the transformation of fat to sugar, then carbohydrate oxidation should not be affected by insulin, the oxygen consumption should fall, the CO_2 production should remain constant, the R.Q. should rise, and the rate of disappearance of blood sugar should be increased to the normal figure because of the cessation of sugar production.

If, however, there is no transformation of fat to sugar in the heart and there is a failure of carbohydrate oxidation in the diabetic heart the heart would perforce have to oxidize fat directly, in which case its R.Q. would be 0·70, then if insulin controls carbohydrate oxidation, it should restore this lost power, increase the rate of disappearance of blood sugar, and thus raise the R.Q. to unity.

From experiments carried out in this laboratory it would appear that the isolated diabetic heart which has a R.Q. of 0·70, upon the addition of insulin to the perfusing blood, increases its R.Q. to unity, increases the rate of disappearance of blood sugar from approximately 4–8 mg./g./hour, very slightly increases its oxygen consumption, namely about 4 p.c., and shows a very high glycogen content which averages 1·39 g./100 g. This latter figure, however, does not indicate any marked increase in glycogen synthesis, as the diabetic heart has, in the diabetic animal, a high glycogen content and thus has its carbohydrate stores surcharged.

The following table indicates the type of result obtained where 5 units of insulin have been given at the beginning of the second period.

Period 30 min.	CO_2 c.c.	O_2 c.c.	R.Q.	Sugar	Fat	Calories per g.
1	132·34	182·20	0·72	—	0·09	0·0120
2	183·24	186·30	0·98	0·24	—	0·0132
3	147·70	190·14	0·77	0·04	0·08	0·0130

Weight of heart = 70 g.

The sugar equivalent of 186·30 c.c. O_2 is 0·248 g.; the amount of blood sugar lost during the second period is 0·265 g. and the glycogen content of the heart is 1·45 g./100 g.

If over-production obtains and its control is the only function of insulin, then insulin should increase the rate of sugar disappearance to the normal, which is 5 mg./g./hour. But here the rate of disappearance is increased not up to but far beyond the normal, namely to 7·10 gm./g./hour. The change in oxygen consumption is almost negligible, while that in CO_2 production shows a marked increase.

It is clear then that such results would indicate that the isolated diabetic heart has under the influence of insulin made an almost equicaloric change from fat to carbohydrate, a fact which is in agreement

with the results obtained by Cori and Cori [1928] on the effect of insulin on the heat production of rats in the post-absorptive state.

The fact that the diabetic heart is so thoroughly charged with glycogen explains why the synthetic activity so characteristic of insulin in the normal heart is here not so markedly in evidence.

While the argument set forth upon these results does not by any means rule out of consideration the theory of the production of sugar from fat, yet, in conjunction with the findings on the metabolism of the heart muscle in aglycæmia, they would certainly tend to weight the balance of experimental evidence in favour of the theory that a failure in the oxidation of carbohydrate is the primary defect in pancreatic diabetes.

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The *p*H of the arterial blood during gastric secretion. By C. E. Brunton. (*School of Medicine, the University of Leeds.*)

The combination of the electrometer valve circuit of Platt and Winfield [1933] with a Haber form of glass electrode and a warm air bath with thermostatic regulation which can contain the arm of the subject, two operators, the electrodes and various solutions [Dickinson, Havard and Platt, 1933], has made possible a higher degree of accuracy in *p*H readings on arterial blood than has hitherto been applied to a study of possible changes in the blood during gastric secretion. Four experiments are reported which were made on the writer through the kindness of Dr Platt, Prof. McSwiney and their colleagues. The *p*H determinations in the first two were made by B. S. Platt and S. Dickinson, those in the third and fourth by S. Dickinson and R. E. Havard. Precautions to be observed are detailed in the article of S. Dickinson and B. S. Platt [1933]. The measurements of *p*H were made at 37° C. The constancy of the apparatus was checked by a known buffer solution before and after each blood sample. An accuracy of $\pm$ 0·01 *p*H unit is claimed for the figures given. R. E. Havard obtained blood by arterial puncture in all the experiments. E. M. Killick kindly estimated the CO_2 content of the blood in the latter two experiments. The other analyses were carried out by the writer.

The stomach was first emptied and a beef extract, dissolved in hot water, was taken as a gastric stimulant. Samples of the stomach contents, 10–15 c.c. in volume, were removed at intervals of 30 min. The first sample

was removed within a few minutes so as to ensure that any increase of acidity found later would be due to a genuine secretion from the gastric glands and not to the introduction of an acid fluid.

TABLE I.

Exp.	Stomach contents Max. free acidity (c.c. 0·1 *N* acid per 100 c.c.)	*p*H of arterial blood (±0·01 unit) Before meal	Time after max. acidity (min.)	During period of secretion	Diff.	CO_2 of arterial blood (c.c./100 c.c. blood) Before meal	During period of secretion
1	49·0	7·382	48	7·380	−0·002	—	—
2	62·0	7·365	10	7·430*	+0·065*	—	—
3	61·5	7·409	4	7·425	+0·016	48·9	47·6
4	52·5	7·380	35	7·405	+0·025	49·3	49·6

* = doubtful result.

Results are given in Table I. The times at which the second blood samples were taken, measured from the middle of the 30 min. period of maximal gastric acidity, are represented in column 4. The *p*H of the second sample in Exp. 2 may be incorrect owing to incomplete filling of the electrode with the blood. The results indicate that there was little change in the *p*H of the subject's arterial blood following meals of meat extract which produced a reasonably large gastric secretion.

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The influence of nucleic acid derivatives upon the coronary blood flow in the dog. By A. N. DRURY and A. M. WEDD.

The dilator action of the nucleic acid derivatives upon the coronary vessels has already been described. These substances, however, when injected into the intact animal, bring about a sharp fall in general blood-pressure and for this reason it has been thought desirable to observe the coronary flow in relation to the general blood-pressure.

The method used in these observations was essentially that of Morawitz and Zahn. Morphia and chloralose were employed as an anæsthetic and chlorazol as an anti-coagulant.

The following substances were tested: adenosine, muscle adenylic acid, yeast adenylic acid and yeast cytidylic acid. In every instance the coronary flow was increased, regardless of the fall in general blood-

pressure. In some instances, when the dose employed failed to influence the general blood-pressure, an appreciable increase in the coronary flow was observed. Of the substances tested yeast adenylic acid produced the greatest increase in flow for a given dose and had the longest duration (3–4 min.). The action of cytidylic acid was very similar. Muscle adenylic acid and adenosic gave rise to considerable increases in flow, but of a distinctly shorter duration (1½ min.). At the time of maximum flow an increase of 150–250 p.c. was usually recorded.

The effect of vitamin B_1 upon the respiratory quotient of brain. By H. M. Sinclair. (*Department of Biochemistry, Oxford.*) (*Preliminary communication.*)

The R.Q. of minced cerebral tissue of polyneuritic pigeons has been determined with the apparatus described by Dixon and Keilin [1933]. In Ringer-phosphate solution, containing 0·033 *M* lactate, *p*H 7·30, the R.Q.'s are uniformly low (0·58–0·78). The determinations have been made over a period of 1 hour in oxygen, at 38° C. Upon adding crystalline vitamin B_1 concentrates [Kinnersley, O'Brien and Peters, 1933], in amount 1 to 2γ per 3 c.c., a consistent rise in the R.Q. is obtained (0·76–1·0). The R.Q. of the extra metabolism produced by the addition of the vitamin is of the order of 1·7. Under identical conditions, cerebral tissue of normal pigeons gives an R.Q. of about 0·88.

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